## REMARKS

Applicants acknowledge with appreciation the telephonic interview with the Examiner on April 3, 2007, during which the foregoing claim amendments and following rejections were discussed.

Claims 13-16 and 26-31 were pending in the application. Claim 13 has been amended and new claim 32 has been added. Accordingly, upon entry of the amendments presented herein, 13-16 and 26-32 will remain pending in the application.

Claim 13 has been amended to specify a method for diagnosing an autoimmune disease, by taking a sample from a patient and testing the sample for IgA antibodies against human tissue transglutaminase and at least one other transglutaminase molecule selected from the group consisting of a-subunit of factor XIII (FXIIIA), keratinocyte transglutaminase (TGk), transglutaminase X (TGx), epidermal transglutaminase (TGe) and Band 4.2, wherein the presence of IgA antibodies in the sample is indicative of the existence of a gluten sensitive enteropathic autoimmune disease, thereby diagnosing an autoimmune disease. Support for this amendment can be found throughout the specification and claims as originally filed. Specifically, support is available at page 2, lines 5-9; page 2, line 29 through page 3, line 26; and page 20, lines 19 through page 22, line 17.

New claim 32 encompasses the method of claim 13 and specifies that the presence of IgA antibodies against at least one other transglutaminase molecule selected from the group consisting of a-subunit of factor XIII (FXIIIA), keratinocyte transglutaminase (TGk), transglutaminase X (TGx), epidermal transglutaminase (TGe) and Band 4.2 is indicative of the presence of a second autoimmune disease. Support for this amendment can be found throughout the specification and claims as originally filed. Specifically, support is available at page 2, lines 5-9; page 2, line 29 through page 3, line 26; and page 20, lines 19 through page 22, line 17.

Claims 15-16 and 26-31 have been withdrawn. However, as acknowledged by the Examiner, Applicants will be entitled to consideration of claims to additional species which are written in dependent form or otherwise include all the limitations of an allowed generic claim as provided by 37 C.F.R. § 1.141. It is further Applicants' understanding that the species election was for search purposes only and that the search will be extended to additional species upon a finding of allowable subject matter.

*No new matter has been added.* Any amendment and/or cancellation of the claims should in no way be construed as an acquiescence to any of the Examiner's rejections and was performed solely in the interest of expediting prosecution of the application. Applicants reserve the right to pursue the claims as originally filed in this or a separate application(s).

***Objections to the Specification***

*Reference to Claims*

At paragraph 2 of the present Office Action, the Examiner has objected to the reference to claims in the specification as being improper because "the claim numbering is subject to change throughout prosecution and thus can cause discrepancies between the specification and the claims."

Applicants have amended the specification to delete reference to particular claims, thereby rendering this objection moot. Accordingly, Applicants respectfully request that the Examiner reconsider and withdraw this objection.

*Typographical Errors*

At paragraphs 3 and 4 of the present Office Action, the Examiner has objected to the specification as containing typographical errors.

Responsive to the Examiner's rejection, Applicants have corrected the typographical error noted by the Examiner, as well as several others. As such, this rejection should now be moot.

***Rejection of Claims 13-14 Under 35 U.S.C. § 112, First Paragraph - Enablement***

The Examiner has rejected claims 13-14 under 35 U.S.C. § 112, First Paragraph, because, according to the Examiner,

> the specification, while being enabling for a method of diagnosing gluten sensitive enteropathy by testing a sample for IgA antibodies directed against human tissue transglutaminase and TGe and correlating increased amounts of the IgA antibodies with a diagnosis of gluten sensitive enteropathy, does not reasonably provide enablement for any and all GSE-type or associated with gluten sensitive enteropathy or any and all antibodies for differential diagnosis of FXIIIA, TGk, TGx and Band 4.2 as broadly recited.

Applicant's respectfully traverse the Examiner's assertion that "the specification does not show any and all antibodies to diagnose as recited." However, to expedite prosecution and allowance of the pending claims, Applicants have amended claim 13 to specify "IgA antibodies," thereby rendering this rejection moot. As such, Applicants respectfully request that the Examiner withdraw the foregoing rejection.

Further, the Examiner questions how one of ordinary skill art can differentiate coleiac disease (CD) from dermatitis herpetiformis (DH) since the specification (*i.e.*, Figure 6), allegedly teaches that both appear to have the same amount of antibodies.

Applicants respectfully traverse the foregoing rejection. Claim 13, as amended, and claims depending therefrom, are drawn to methods for *diagnosing an autoimmune disease*, by taking a sample from a patient and testing the sample for IgA antibodies against human tissue transglutaminase and at least one other transglutaminase molecule selected from the group consisting of a-subunit of factor XIII (FXIIIA), keratinocyte transglutaminase (TGk), transglutaminase X (TGx), epidermal transglutaminase (TGe) and Band 4.2, *wherein the presence of IgA antibodies in the sample is indicative of the existence of a gluten sensitive enteropathic autoimmune disease*, thereby diagnosing an autoimmune disease.

Contrary to the Examiner's interpretation, the object of the present invention is not to differentiate coleiac disease (CD) from dermatitis herpetiformis (DH), but instead is to determine whether an autoimmune disease is, in part, caused by a sensitivity to gluten. Applicants acknowledge that the etiologies of the autoimmune diseases recited by Applicants at, for example, page 2, Table 2 of Applicants' specification, are not fully known and/or understood. However, it is the object of the invention to provide a method for determining the etiology of an autoimmune disease, so as to enable treatment of the disease. Accordingly, the methods of the present invention are useful for further testing a suspected or known autoimmune disease to provide a better means for diagnosis of the true nature and cause of the autoimmune disease under investigation.

The Examiner also asserts that claims 13 and 14 lack enablement because, according to the Examiner,

> [t]he specification does not provide a definition for GSE-type nor does the specification specifically teach how diseases are associated with gluten sensitive enteropathy. The specification on page 21 discloses in Table 2 a list of autoimmune diseases that have been reported to associate with GSE and states that a part of the associations are proven, the other part have been anecdotal. The specification does not provide guidance on what is a GSE-type autoimmune disease nor does the specification provide relevant association of these diseases for differential diagnosis or provide evidence or data which could provide one of skill in the art how to differentiate one disease listed in table 2 from the other disease.

Applicants respectfully traverse this rejection for those reasons discussed above. However, to expedite prosecution and allowance of the pending claims, claim 13 has been amended so that it no longer recites the phrase "autoimmune diseases of the GSE-type or associated with gluten sensitive enteropathy", thereby rendering this rejection moot. As such, Applicants respectfully request that the Examiner withdraw the foregoing rejection.

The Examiner further asserts that claims 13 and 14 lack enablement because the Examiner is of the opinion that "[t]he specification also fails to provide any evidence concerning the presence and significance of auto-antibodies specific for or detectable by transglutaminases

known as TGk, TGx, Factor XIIIA or band 4.2. There are no working examples provided in the specification."

Applicants respectfully traverse the Examiner's assertion and respectfully submit that the specification does indeed provide evidence concerning the presence and significance of auto-antibodies specific for or detectable by transglutaminases known as TGk, TGx, Factor XIIIA or band 4.2. Specifically, Applicants teach "the discovery of TGc as the main autoantigen in GSE did not answer the question why only a proportion of patients with CD also show symptoms of DH and if...there is a difference in the antigenic repertoire in these diseases." Further, the discovery that "immunostaining for TGc does not give the same staining pattern...for IgA precipitates in the skin of DH patients" led Applicants to believe that antigen in DH patients might be different for human tissue transglutaminase. Therefore, as part of the present invention, Applicants "expressed three other transglutaminases which can be found in the skin, the human TgH, TGe and TGx, in human embryonic kidney cells" to further investigate whether an autoimmune disease may, in part, be caused by a sensitivity to gluten.

Finally, Applicant's respectfully traverse the Examiner's assertion that "there are no working examples provided in the specification" and submit that the amount of direction and guidance provided in the specification is more than sufficient to enable one of ordinary skill in the art to make and use the presently claimed invention. Specifically, Applicants' invention is based on the novel discovery that transglutaminases, other than human tissue transglutaminase, play a role in autoimmune diseases and can be used to diagnose such diseases. Moreover, Applicants teach the production of expression constructs for the expression of TGc, TGe, TGx and TGk, as well as recombinant expression of these transglutaminases in human embryonic kidney cell (see page 7, line 6 through page 10, line 12 of the specification). Applicants also teach ELISA assays which can be used to test a sample for IgA antibodies against human tissue transglutaminase and at least one other transglutaminase (see page 12, lines 1-31 and page 15, line 4 through page 17, line 6). Accordingly, based on the teachings of the specification, in

combination with knowledge available in the art, one of ordinary skill in the art would be able to generate and express the transglutaminases encompassed by the claimed methods and assay a given sample for reactivity with a specific transglutaminase, without undue experimentation, to determine whether an autoimmune disease is caused, in part, by a gluten sensitivity.

In view of the foregoing, Applicants respectfully submit that the specification provides more than sufficient guidance to enable one of ordinary skill in the art to make and use the presently claimed invention. As such, Applicants respectfully request that the Examiner reconsider and withdraw the foregoing rejection.

***Rejection of Claims 13-14 Under 35 U.S.C. § 112, Second Paragraph***

The Examiner has rejected claim 13 under 35 U.S.C. § 112, second paragraph as being indefinite because the preamble of the claim does not correlate with the body of the claim, *i.e.*, the body of the claim does not positively recite a step of differentially diagnosing autoimmune diseases of the GSE-type of associated with gluten sensitive enteropathy.

Responsive to the Examiner's rejection, Applicants have amended claim 13 to recite a positive method step, thereby rendering this rejection moot.

Claim 13 is further rejected because, according to the Examiner, the term "GSE-type" is vague and indefinite.

Applicants respectfully traverse this rejection. However, to expedite prosecution, the claims, as amended, no longer recite the term "GSE-type." Accordingly, this rejection is now moot and Applicants respectfully request that the Examiner withdraw the foregoing rejection.

The Examiner has also rejected claim 13 as being vague and indefinite because, according to the Examiner, "[t]he specification does not provide a definition for the term 'associated'"and "it is unclear how autoimmune diseases are associated with gluten sensitive enteropathy."

With respect to the Examiner's assertion that "it is unclear how autoimmune diseases are associated with gluten sensitive enteropathy," Applicants acknowledge that the etiologies of the autoimmune diseases encompassed by the pending claims are not fully known and/or understood, as discussed above. However, it is the object of the invention to provide a method for differentiation and, subsequently, for determining the etiology of an autoimmune disease, so as to enable treatment of the diseases. Accordingly, the methods of the present invention are useful for further testing a suspected or known autoimmune disease to provide a better means for diagnosis of the true nature and cause of the autoimmune disease under investigation.

Notwithstanding, to expedite prosecution and allowance of the pending claims, claim 13 no longer recited the phrase "associated", thereby rendering this rejection moot. As such, Applicants respectfully request withdrawal of this rejection.

Claim 13 is also rejected as being vague and indefinite on the basis that:

> it is unclear how testing the sample for antibodies as recited is correlated to a differential diagnosis. Does the mere presence of antibodies indicate a differential diagnosis? Does an increase in antibodies against human tissue transglutaminase and a decrease of antibodies to the other molecules indicate a differential diagnosis? Does an increase in antibodies to both the human tissue transglutaminase and the other molecule indicate a differential diagnosis? Further, what is being differentially diagnosed? Is a GSE-type disease being differentiated from a disease associated with gluten sensitive enteropathy? Is a GSE-type disease being differentiated from GSE? If so how?

Applicants respectfully traverse the foregoing rejection. Notwithstanding, to expedite prosecution, claim 13 has been amended to clarify that the presence of IgA antibodies in the sample is indicative of the existence of a gluten sensitive enteropathic autoimmune disease. As such, claim 13 is clear and definite and Applicants respectfully request withdrawal of this rejection.

The Examiner has also rejected claim 13 as being vague and indefinite based on the use of acronyms, *i.e.*, GSE, TGK, TGe, *etc.*, and asserts that these terms should be defined in their first instance.

Applicants respectfully traverse the foregoing rejection. Abbreviations, which are commonly used in the art may be recited in a claim without reciting the entire phrase for which the abbreviation is used. Applicants respectfully submit that the abbreviations recited in claim 13, *i.e.*, GSE, FXIIIA, TGk, TGx, and TGe, are all common, art-recognized terms and accordingly, a person skilled in the art, at the time of filing the present application, would understand the meaning of these terms without further definition. For example, as evidenced by Ahmed *et al.*, *J. Exp. Med.*, 1993 Dec 1;178(6):2067-75 (enclosed herewith as Appendix A, the term "GSE" is a standard abbreviation for "gluten sensitive enteropathy" that would have been readily recognized by one of ordinary skill in the art prior to the filing of the present application. Further, as evidenced by Aeschlimann *et al.*, *J Biol Chem.* 1998 Feb 6;273(6):3452-60 (enclosed herewith as Appendix B), the abbreviation "FXIIIA" stands for the a-subunit of factor XIIIa, *i.e.*, a tetrameric protein known for its role in blood clotting, the abbreviation "TGk" stands for keratinocyte transglutaminase, the abbreviation "TGx" stands for transglutaminase X, a transglutaminase that is believed to contribute to the formation of the cornified envelope, and the abbreviation "TGe" stands for epidermal transglutaminase. Accordingly, as demonstrated by Aeschlimann *et al*, FXIIIA, TGk, TGx, and TGe are all standard abbreviations that would have been readily recognized by one of ordinary skill in the art prior to the filing of the present application and as such, Applicants should not be required to further define the abbreviations.

Notwithstanding, to expedite prosecution and allowance of the pending claims, Applicants have amended claim 13 to recite the full terms which correspond to the recited abbreviations. Accordingly, Applicants respectfully request that the Examiner reconsider and withdraw this objection.

Claim 13 is further rejected as being indefinite based on the phrase "taking a sample" because, according to the Examiner, it is "unclear what the sample is being taken from."

Applicants respectfully traverse this rejection. However, to expedite prosecution and allowance of the pending claims, Applicants have amended claim 13 to specify that a sample is taken from "a patient," thereby rendering this rejection moot.

***Rejection of Claim 13 Under 35 U.S.C. § 102***

The Examiner has rejected claim 13 under 35 U.S.C. § 102 as being anticipated by Schuppan *et al.* (US 6,319,726). The Examiner relies on Schuppan *et al.* for teaching "methods of detecting antibodies from body fluids by means of an immune reaction with tissue transglutaminase," such as human tissue transglutaminase. The Examiner also relies on Schuppan *et al.* for teaching "that the tissue transglutaminase can be immobilized and used to detect antibodies in a sample for diagnosing celiac disease (GSE-type, associated with GSE)" and that method "is used to detect IgA antibodies."

The Examiner further asserts that Schuppan *et al.* reads on the claimed limitation of "testing the sample for antibodies against ...at least one other transglutaminase molecule selected from FXIIIA, TGk, TGx, TGe and Band 4.2." Specifically, the Examiner asserts that

> Schuppan et al disclose that the antibodies to be detected are IgA antibodies which are against human tissue transglutaminase. These antibodies to be detected are the same as the antibodies detected by applicant (see specification). Thus, the antibodies of Schuppan et al would be cross reactive with other antigens and would inherently be against TGe. As shown by Applicant the IgA antibodies are cross reactive. The specification on page 15 discloses 'the results show support that serum IgA antibodies from patients with CD and DH react with both human TGc and TGe' and further discloses that the serum antibodies from patients with CD and DH is directed against epitopes which are shared by the two transglutaminases.' Thus it is inherent that the IgA antibodies of Schuppan et al are against both human tissue transglutaminase and TGe.

Applicants respectfully traverse this rejection. For a prior art reference to anticipate a claimed invention under 35 U.S.C. § 102, the prior art must teach *each* and *every* element of the claimed invention invention. *Lewmar Marine v. Barient*, 827 F.2d 744, 3 USPQ2d 1766 (Fed.

Cir. 1987). The presently claimed methods for diagnosing an autoimmune disease include taking a sample from a patient and testing the sample for IgA antibodies against human tissue transglutaminase and *at least one other transglutaminase molecule* selected from the group consisting of a-subunit of factor XIII (FXIIIA), keratinocyte transglutaminase (TGk), transglutaminase X (TGx), epidermal transglutaminase (TGe) and Band 4.2, wherein the presence of IgA antibodies in the sample is indicative of the existence of a gluten sensitive enteropathic autoimmune disease, thereby diagnosing an autoimmune disease. As discussed above, the aim of the present invention is to determine whether an autoimmune disease is caused, in part, by a gluten sensitivity.

In contrast, Schuppan *et al.* teach methods of identifying coeliac disease/non-tropical sprue disease in a subject by detecting antibodies from body fluids by means of an immune reaction with tissue transglutaminase (tTG). Schuppan *et al.* fail to teach or suggest a method which encompasses the detection of antibodies against human tissue transglutaminase *and at least one other transglutaminase molecule selected from group consisting of FXIIIA, TGk, transglutaminase X TGx, TGe and Band 4.2.*

Further, contrary to the Examiner's assertion, the antibodies of Schuppan *et al.* would not necessarily "be cross reactive with other antigens," *i.e.*, such as those antigens encompassed by the present invention. In fact, none of the transglutaminases (*i.e.*, TGk, TGx, TGe) encompassed by the present methods are homologous to human tissue transglutaminase. Therefore, there is no factual to suggest that the antibodies of Schuppan *et al.* would cross-react with TGk, TGx, TGe, nor is there basis to suggest that the antibodies of Schuppan *et al.* would cross-react with FXIIIA or Band 4.2.

Moreover, Applicants respectfully traverse the Examiner's assertion that the antibodies of Schuppan *et al.* would "inherently" cross-react with TGe, in particular. The only common feature shared by human tissue transglutaminase and TGe is that both are transglutaminases, *i.e.*, members of a family of enzymes that catalyze the formation of a covalent bond between a free

amine group and the gamma-carboxamid group of protein- or peptide bound glutamine. Human tissue transglutaminase and TGe are distinct, non-homologous antigens, which have different origins, as well as different primary and secondary sequences. Although Applicants do teach that "serum IgA antibodies from *patients with* [both] *CD and DH* react with both the human TGc and TGe" and that "[a]t least a *part of the serum* antibodies from *patients with* [both] *CD and DH* is directed against epitopes which are shared by the two transglutaminases" (page 15, lines 12-16 of the specification; emphasis added), this does not correlate with the assumption that antibodies against tTG (human tissue transglutaminase) would cross-react with TGe (epidermal tissue transglutaminase). This is because the Applicants test the patient's plethora of various antibodies in the serum only for the presence of antibodies binding to TGe or TGx or FXIIIa, in addition to tTG, and not for cross-reacting antibodies. As noted in the specification, "only a proportion of patients with CD also show symptoms of DH" (page 3, lines 28-30). As such, antibodies from a patient having only CD, *i.e.*, such as those exemplified in Schuppan *et al.*, will not necessarily cross-react with TGe.

Since Schuppan *et al.* fail to teach or suggest each and every element of the present invention, *i.e.*, methods which encompasses the detection of antibodies against human tissue transglutaminase *and at least one other transglutaminase molecule selected from group consisting of FXIIIA, TGk, transglutaminase X TGx, TGe and Band 4.2,* claim 13 is novel over the cited reference. Therefore, Applicants respectfully request the Examiner to reconsider and withdraw this rejection.

## CONCLUSION

In view of the above amendments and remarks set forth above, it is respectfully submitted that this application is in condition for allowance. If there are any remaining issues or the Examiner believes that a telephone conversation with Applicants' Attorney could be helpful in expediting prosecution of this application, the Examiner is invited to call the undersigned at (617) 227-7400.

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Respectfully submitted,

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# Major Histocompatibility Complex Susceptibility Genes for Dermatitis Herpetiformis Compared with Those for Gluten-sensitive Enteropathy

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## Summary

Dermatitis herpetiformis (DH) shares some clinical features and major histocompatibility complex (MHC) markers with gluten-sensitive enteropathy (GSE). We compared MHC haplotypes in 27 patients with DH, 35 patients with GSE, and normal controls. As in GSE, the frequencies of two extended haplotypes, [HLA-B8, SC01, DR3] and [HLA-B44, FC31, DR7], were increased in patients with DH. Distributions of fragments of extended haplotypes, consisting of some but not all of the elements of complete extended haplotypes, were analyzed to attempt to localize a susceptibility gene. Besides complete extended susceptibility haplotypes, (DR3, DQ2) and (DR7, DQ2) fragments were most common in GSE. In contrast, DH showed only a few such fragments but many instances of the fragment (SC01). The differences in distribution of these fragments in the two diseases were highly significant ($P < 0.002$). HLA-DQ2 and DR3 had the highest odds ratios for GSE, but the highest odds ratio for DH was for the complotype SC01. These findings suggest that the MHC susceptibility gene for DH is between class II and complotype regions, closest to the complotype, whereas that for GSE is in the class II region.



Dermatitis herpetiformis (DH)[1] is a chronic pruritic papulo-vesicular disease of the skin characterized by the granular deposition of IgA in the dermal papillae and by patchy focal asymptomatic villous atrophy of the small intestine (1, 2). In Caucasian patients, there is a marked increase in HLA-B8, DR3, and DQ2 (3–7). These genes are components of the fixed conserved extended haplotype [HLA-B8, SC01, DR3] (8). The recent report of an increase in HLA-DP1 (9) in patients with DH who carry markers of this haplotype may be secondary to their known linkage disequilibrium in general (10, 11). A less striking increase in HLA-Dw7 in DH patients has also been reported (12). The same HLA markers have been noted to be increased in celiac disease (gluten-sensitive enteropathy [GSE]) (13–16). Moreover, patients with DH and GSE share a number of other features, such as jejunal atrophy correctable by elimination of gliadin from the diet, and the presence of anti-α-gliadin (17, 18) and antireticulin (19, 20) antibodies. We have reported family studies in GSE (21) that show that all increased available HLA markers are parts of two extended haplotypes: [HLA-B8, SC01, DR3] and [HLA-B44, FC31, DR7].

Previous studies of DH have almost exclusively reported HLA phenotypes in patients rather than haplotypes determined in family studies. Haplotype comparisons, including analysis for complotypes and other MHC genes, are critical to localizing candidate susceptibility genes, and we undertook such studies in patients with DH. We present these results in this report and compare these DH haplotypes with those derived from our earlier studies in GSE. Our results suggest that, despite the similarity of individual MHC allele markers for GSE and DH, the susceptibility genes for the two disorders are different. That for GSE appears to be in or near the HLA-DR/DQ region, whereas that for DH appears to be between complement and DR/DQ, closest to the complement region.

[1] *Abbreviations used in this paper:* DH, dermatitis herpetiformis; GSE, gluten-sensitive enteropathy.

## Materials and Methods

*Samples.* Blood from 27 randomly ascertained Caucasian patients with dermatitis herpetiformis and 147 of their first-degree relatives was collected into 7-ml Vacutainer tubes (Becton Dickinson & Co., Rutherford, NJ) containing 10.5 mg of potassium EDTA and into syringes containing 500–1,000 U of sodium heparin diluted with an equal volume of RPMI 1640. 25 patients were unrelated; 2 (43416 and 43417) were siblings. Lymphocytes were separated from heparinized blood by Ficoll-Hypaque centrifugation, frozen, and stored in vapor phase liquid nitrogen until thawed for HLA analysis. Plasma separated from blood anticoagulated with EDTA was stored at −80°C until just before analysis for complement types.

*Patients.* All patients with DH had a history of and on physical examination had evidence of typical pruritic grouped vesicles on extensor surfaces of limb skin. The diagnosis of DH was confirmed by the presence of granular deposits of IgA by direct immunofluorescence microscopy in the dermal papillae of perilesional skin (22). 35 patients with GSE were studied, of which 24 were reported earlier (21).

*MHC Marker Studies.* HLA-A, B, DR, and DQ typing were by standard assays (23, 24). Plasma samples were analyzed for genetic polymorphism in C2 (25), in factor B (BF) (26), and in C4 (27) by methods previously described. Complotypes are haplotypes of specific alleles of these four closely linked loci and are designated in arbitrary order by their BF, C2, C4A, and C4B alleles, including Q0 for null alleles (28). Thus, FC30 stands for *BF*F, C2*C, C4A*3,* and *C4B*Q0.*

*Haplotype Analysis.* Alleles were assigned to haplotypes on the basis of family studies except for sequence-specific oligonucleotide-determined alleles, which were studied only in patients. The two DH-affected siblings were MHC haploidentical and thus contributed three independent disease haplotypes to the analysis.

*Class II (HLA-DR, DQ, and DP) Allele Typing.* Genomic DNA was isolated from PBL or from EBV-transformed cell lines of 17 DH patients and 10 GSE patients. Sequence-specific oligonucleotide (SSO) probe hybridization was carried out according to the protocols of the 11th International Histocompatibility Workshop (11). Briefly, PCR amplifications were carried out on 0.6-$\mu$g samples of purified genomic DNA in a 100-$\mu$l reaction mixture containing 50 pmol of each primer, 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 0.2 mM of each deoxynucleotide, 2.5 U of *Taq* polymerase (Promega Biotec, Madison, WI), and 1.5 mM $MgCl_2$. The primers used were those recommended by the 11th Workshop. About 50 ng of amplified DNA was spotted on several nylon membranes. These were prehybridized at 54°C with hybridization buffer (50 mM Tris HCl, 3 M tetramethyl ammonium chloride, 2 mM EDTA, 0.1% SDS, 100 mg/ml heat-denatured herring sperm DNA, and 5× Denhardt's solution) for 1 h. The allele-specific oligonucleotides labeled with $\gamma$-[$^{32}$P]ATP were added. HLA-DRB1, -DQA1, -DQB1, and -DPB1 alleles were identified after incubation, washing, and autoradiography of the exposed membranes. Haplotype assignments were deduced from known linkage disequilibria for all loci but DPB1. The order of genes in the MHC from telomere to centromere is HLA-A, B, complotype, DR, DQ, and DP.

*Statistical Analyses.* Statistical significance of the differences in frequency of individual MHC alleles and extended haplotypes in the patient and control populations of haplotypes was estimated by $\chi^2$ analysis or by Fisher's exact test, as appropriate. Since prior work has shown elements of [HLA-B8, SC01, DR3, DQ2] and [HLA-B44, FC31, DR7, DQ2] to be elevated among patients, no corrections were applied to $p$ values for significance of differences in frequency from normals. Two kinds of control haplotypes were used, family and overall Caucasian controls (29).

## Results

*MHC Haplotypes in DH.* MHC haplotypes occurring in 27 patients with DH are shown in Table 1. All but nine haplotypes contained elements of one or the other of two extended conserved MHC haplotypes, [HLA-B8, SC01, DR3] or [HLA-B44, FC31, DR7]. The patient haplotypes were arranged in Table 1 in relation to these extended haplotypes and their fragments from telomeric to centromeric. Two haplotypes, 42453b and 43655b, had elements of both extended haplotypes and are shown in each category. The arrangement permits the easy identification of fragments of the extended haplotypes. Thus, there were 22 instances of the complete [HLA-B8, SC01, DR3] extended haplotype and 10 examples of fragments of this haplotype. Of the fragments, there were two examples of (SC01, DR3) without B8, and single examples of B8 without SC01 or DR3, (B8, SC01) without DR3, and DR3 without SC01. In contrast, there are five cases of (SC01) without B8 or DR3. Because HLA-DQ2, in strong linkage disequilibrium with both DR3 and DR7, was also increased markedly (see below), these findings suggest that the susceptibility gene lies between the complotype region and HLA-DR/DQ.

Of the 16 haplotypes related to [HLA-B44, FC31, DR7], there were 3 instances of the complete extended haplotype. Of the 13 cases of fragments, 5 had HLA-B44 and complotypes other than FC31, 6 had DR7 but not FC31 (2 of these had SC01), 2 had (FC31, DR7) but not HLA-B44, there was 1 instance of (FC31) alone, and 6 with (DR7, DQ2) alone. These findings are consistent with the possibility that a susceptibility gene lies in the complotype-DR/DQ region of the MHC. In keeping with these conclusions was the finding that all (27/27) patients carried SC01 or FC31 and almost all carried HLA-DQ2 (26/27) and DR3 or DR7 (26/27).

*MHC Class II Alleles Defined by Sequence-specific Oligonucleotide Typing.* HLA-DRB1, DQA1, and DQB1 alleles are listed in Table 2 for patients with DH. In all instances, the DQA1 and DQB1 alleles present conformed to the DRB1 alleles predicted from known essentially invariable linkage disequilibria in Caucasians. Of the 27 DH patients, 21 carried the DRB1*0301, DQA1*0501, DQB1*0201 haplotype found on all HLA-DR3 haplotypes and instances of [HLA-B8, SC01, DR3]. Of the six patients who did not, three were DRB1*11, DQA1*0501, DQB1*0301/DRB1*07, DQA1*0201, DQB1*-0201 heterozygotes, and one each were heterozygotes for DRB1*0103, DQA1*0101, DQB1*0501/DRB1*07, DQA1*-0201, DQB1*0201; DRB1*0101, DQA1*0101, DQB1*0501/DRB1*04, DQA1*0301, DQB1*0302, and DRB1*04, DQA1*-0301, DQB1*0302/DRB1*07, DQA1*0201, DQB1*0303. The overall distribution of DR specificity homozygotes and heterozygotes was not different from that predicted from the Hardy-Weinberg distribution.

The frequency of HLA-DPB1*0101 (6/28) was increased ($p < 0.003$) among DH haplotypes compared with the fre-

**Table 1.** *MHC Haplotypes in Patients with DH*

| Haplotype No. | HLA-A | HLA-B | Complotype | HLA-DR |
|---|---|---|---|---|
| Haplotypes related to [HLA-B8, SC01, DR3]: | | | | |
| 42306a | 2 | 8 | SC32 | 4 |
| 41019a | 1 | 8 | SC01 | 3 |
| 42290a | 1 | 8 | SC01 | 3 |
| 42372c | 1 | 8 | SC01 | 3 |
| 42388d | 1 | 8 | SC01 | 3 |
| 42394d | 1 | 8 | SC01 | 3 |
| 42439a | 1 | 8 | SC01 | 3 |
| 42445c | 1 | 8 | SC01 | 3 |
| 42447a | 1 | 8 | SC01 | 3 |
| 43342b | 1 | 8 | SC01 | 3 |
| 43406a | 1 | 8 | SC01 | 3 |
| 43411a | 1 | 8 | SC01 | 3 |
| 43411c | 1 | 8 | SC01 | 3 |
| 43416a | 1 | 8 | SC01 | 3 |
| 43998a | 1 | 8 | SC01 | 3 |
| 44026b | 1 | 8 | SC01 | 3 |
| 42399a | 2 | 8 | SC01 | 3 |
| 43352a | 2 | 8 | SC01 | 3 |
| 42306b | 2 | 8 | SC01 | 3 |
| 42301a | 3 | 8 | SC01 | 3 |
| 42394c | 11 | 8 | SC01 | 3 |
| 43342a | 24 | 8 | SC01 | 3 |
| 42338a | 25 | 8 | SC01 | 3 |
| 43655b | 11 | 8 | SC01 | 7* |
| 43417d | 2 | 7 | SC01 | 3 |
| 43376c | 11 | 27 | SC01 | 3 |
| 42312b | 2 | 49 | SC01 | 4 |
| 43902c | 2 | 49 | SC01 | 4 |
| 42447c | 3 | 7 | SC01 | 1 |
| 42453b | 2 | 13 | SC01 | 7* |
| 42359b | 2 | 61 | SC01 | 5 |
| 42453a | 1 | 7 | SC31 | 3 |
| Haplotypes related to [HLA-B44, FC31, DR7]: | | | | |
| 41019c | 2 | 44 | SC30 | 1 |
| 42290c | 24 | 44 | FC30 | 6 |
| 42297c | 2 | 44 | SC31 | 5 |
| 42439b | 32 | 44 | FC(3,2)0 | 1 |
| 43998b | 28 | 44 | SC31 | 7 |
| 42359a | 23 | 44 | FC31 | 7 |
| 43376a | 26 | 44 | FC31 | 7 |
| 42372d | 11 | 44 | FC31 | 7 |
| 42297d | 3 | 7 | FC31 | 7 |
| 43398a | 2 | 7 | FC31 | 7 |
| 42388c | 2 | 57 | FC31 | 5 |
| 43352b | 3 | 47 | FC91,0 | 7‡ |
| 42453b | 2 | 13 | SC01 | 7* |
| 42312a | 1 | 57 | SC61 | 7‡ |
| 42338c | 2 | 13 | SC31 | 7 |
| 43655b | 11 | 8 | SC01 | 7* |
| Other haplotypes: | | | | |
| 43406c | 23 | 7 | SC31 | 1 |
| 43902a | 11 | 56 | SC31 | 1 |
| 43416c | 3 | 7 | SC61 | 2 |
| 42301b | 3 | 7 | SC31 | 2‡ |
| 42399d | 25 | 18 | S042 | 2‡ |
| 44026a | 1 | 60 | SC31 | 4 |
| 43655a | 2 | 51 | SC30 | 5 |
| 43398c | 1 | 35 | SC42 | 6 |
| 42445a | 3 | 35 | SC42 | 6 |

Shown are all specificities assigned to haplotypes by family study.
* Listed twice as related to both [HLA-B8, SC01, DR3] and [HLA-B44, FC31, DR7].
‡ Extended haplotypes other than those above.

quency of 3 of 105 among random normal Caucasian haplotypes in our laboratory. 2 of the 3 DPB1*0101 among control chromosomes were carried by [HLA-B8, SC01, DR3] and 18 of 52 [HLA-B8, SC01, DR3] homozygous typing cell haplotypes carried DPB1*0101 ($p < 2 \times 10^{-7}$), indicative of strong linkage disequilibrium between DPB1*0101 and [HLA-B8, SC01, DR3]. Six of six instances of the DPB1*0101 allele occurred in patients who carried DR3 haplotypes, of which five were the complete extended haplotype [HLA-B8, SC01, DR3] and one was the (SC01, DR3) fragment.

*MHC Specificity and Haplotype Frequencies in Patients with DH Compared with Control Haplotypes.* Table 3 gives the frequencies in patients with DH of MHC alleles and extended

**Table 2.** *HLA-DRB1, DQA1, DQB1, and DPB1 Alleles in Patients with DH*

| Patient | First haplotype | | | Second haplotype | | | |
|---|---|---|---|---|---|---|---|
| | DRB1 | DQA1 | DQB1 | DRB1 | DQA1 | DQB1 | DPB1 |
| 43902 | 0101 | 0101 | 0501 | 04 | 0301 | 0302 | ND |
| 43342 | 0301 | 0501 | 0201 | 0301 | 0501 | 0201 | ND |
| 43411 | 0301 | 0501 | 0201 | 0301 | 0501 | 0201 | ND |
| 43406 | 0301 | 0501 | 0201 | 0101 | 0101 | 0501 | 0101/1201 |
| 42439 | 0301 | 0501 | 0201 | 0101 | 0101 | 0501 | 0301/0401 |
| 42399 | 0301 | 0501 | 0201 | 1501 | 0102 | 0602 | 0101/1901 |
| 43416 | 0301 | 0501 | 0201 | 1501 | 0102 | 0602 | 0401/0401 |
| 42306 | 0301 | 0501 | 0201 | 04 | 0301 | 0201 | 0101/0301 |
| 43998 | 0301 | 0501 | 0201 | 07 | 0201 | 0201 | 0101/1101 |
| 42447 | 0301 | 0501 | 0201 | 07 | 0201 | 0201 | 0301/0401 |
| 43376 | 0301 | 0501 | 0201 | 07 | 0201 | 0201 | 0101/1001 |
| 42453 | 0301 | 0501 | 0201 | 07 | 0201 | 0201 | 0401/0501 |
| 43352 | 0301 | 0501 | 0201 | 07 | 0201 | 0201 | 0101/1501 |
| 43398 | 07 | 0201 | 0201 | 0103 | 0101 | 0501 | 0301/0402 |
| 42312 | 07 | 0201 | 0303 | 04 | 0301 | 0302 | 0401/0401 |
| 42359 | 07 | 0201 | 0201 | 11* | 0501 | 0301 | 0301/0401 |
| 43655 | 07 | ND | 0201 | 11* | ND | 0301 | 0401/0701 |

DRB1-DQA1-DQB1 haplotype assignments deduced from known linkage disequilibria. Because linkage relationships of DR,DQ-DPB1 alleles are weaker, no assignments were made.
* 1101 or 1104.

haplotypes of interest or those showing significant differences from the frequencies in control haplotypes. There were striking increases in the frequencies of the extended conserved MHC haplotype [HLA-B8, SC01, DR3] and all of its elements, including HLA-B8, SC01, C4A*Q0, HLA-DR3, and HLA-DQ2.

*Comparison of MHC Haplotypes in Patients with DH with Those in Patients with GSE.* Table 4 lists MHC haplotypes in 35 patients with GSE. In the two groups of patients, only HLA-A29 was higher in GSE ($p = 0.012$) and only HLA-DR1 was higher in DH ($p = 0.018$) among all alleles and extended haplotypes compared. These differences were only nominally significant and lost significance after correction for the number of comparisons. HLA-DRB1, DQA1, and DQB1 alleles observed among GSE patients were those predicted by known linkage disequilibria with generic DR types.

Because so many of the DH patient haplotypes were related to [HLA-B8, SC01, DR3], analyses of the distribution of alleles and haplotypes were carried out after the removal of the complete [HLA-B8, SC01, DR3] haplotype from all patient and control haplotypes. The increases in patients' SC01, C4A*Q0, and HLA-DQ2 remained highly significant. In addition, the increase in [HLA-B44, FC31, DR7] among DH patient haplotypes was just significant (compared with family controls but not to overall controls) at $p < 0.05$. By themselves, these observations suggested that the chromosomal region between SC01 and HLA-DQ2 contains susceptibility gene(s) for DH.

Odds ratios for MHC alleles and haplotypes in the DH and GSE patient and control populations with and without patients positive for the complete [HLA-B8, SC01, DR3] extended haplotype were calculated and the results are given in Table 5. It is seen that for DH the highest odds ratio with and without the extended haplotype was that of SC01 followed by that of HLA-DQ2, whereas in GSE it was DQ2 followed by DR3.

The distribution of fragments of [HLA-B8, SC01, DR3] among 55 DH patient haplotypes (Table 1) was compared with those among 70 GSE patient haplotypes (Table 6). In DH, there were six haplotypes with SC01 but not DR3 and only one haplotype with DR3 but not SC01, whereas in GSE there were no haplotypes with SC01 but not DR3 and eight haplotypes with DR3 but not SC01 ($p < 0.002$), as shown in Table 6.

We had earlier noted that in patients with GSE, HLA-A1 was less frequently found on [HLA-B8, SC01, DR3] than on control haplotypes (Table 4). This phenomenon was not seen in patients with DH (Table 1), in whom 16 of 22 in-

**Table 3.** *Comparison of MHC Allele Frequencies in Patients with DH with Controls*

| Allele | Patient frequency | | Family control frequency | | Normal control frequency | | pt vs. fc | pt vs. nc |
|---|---|---|---|---|---|---|---|---|
| | No. | Fraction | No. | Fraction | No. | Fraction | | |
| | | | | | | | *p* | *p* |
| HLA-A1 | 19 | 0.352 | 11 | 0.190 | 378 | 0.174 | <0.05 | 0.0010 |
| HLA-B8 | 24 | 0.444 | 4 | 0.069 | 265 | 0.122 | <0.0001 | 0.0001 |
| HLA-B15 | 0 | 0.000 | 5 | 0.086 | 121 | 0.056 | <0.04 | NS |
| HLA-DR2 | 3 | 0.056 | 7 | 0.121 | 354 | 0.162 | NS | <0.02 |
| HLA-DR3 | 23 | 0.426 | 7 | 0.121 | 290 | 0.133 | 0.0001 | 0.0001 |
| HLA-DR4 | 4 | 0.074 | 6 | 0.103 | 373 | 0.171 | NS | <0.02 |
| C4A*Q0 | 29 | 0.537 | 7 | 0.121 | 360 | 0.165 | 0.0001 | 0.0001 |
| C4A*2 | 0 | 0.000 | 1 | 0.017 | 133 | 0.061 | NS | <0.04 |
| C4A*3 | 18 | 0.333 | 37 | 0.638 | 1,319 | 0.605 | 0.0010 | 0.0001 |
| C4B*1 | 45 | 0.833 | 37 | 0.638 | 1,530 | 0.702 | 0.02 | NS |
| SC01 | 29 | 0.537 | 6 | 0.103 | 293 | 0.134 | 0.0001 | 0.0001 |
| SC31 | 8 | 0.148 | 19 | 0.328 | 820 | 0.376 | <0.03 | 0.0001 |
| [B8,SC01,DR3] | 22 | 0.407 | 3 | 0.052 | 192 | 0.088 | <0.0001 | 0.0001 |
| [B44,FC31,DR7] | 3 | 0.056 | 0 | 0.000 | 63 | 0.029 | NS | NS |
| Total | 54 | | 58 | | 2,180 | | | |
| DQ1 | 10 | 0.208 | 21 | 0.396 | 192 | 0.420 | <0.05 | 0.004 |
| DQ2 | 31 | 0.646 | 17 | 0.321 | 109 | 0.239 | 0.001 | 0.0001 |
| DQ3 | 7 | 0.146 | 15 | 0.283 | 155 | 0.339 | NS | 0.006 |
| Total | 48 | | 53 | | 457 | | | |

Only alleles or haplotypes of interest or showing significant differences in frequency among the populations of haplotypes are shown.

stances carried HLA-A1, a similar fraction as in normal [HLA-B8, SC01, DR3] haplotypes ($p$ = NS).

## Discussion

Our observations strongly suggest that the major susceptibility genes within the MHC for GSE and DH are different, or at least has different locations, in spite of the fact that the two disorders share clinical features and similar MHC markers, including extended haplotypes. That for GSE appears to lie in the DR/DQ region, whereas that for DH is near the complotype region, perhaps between it and DR/DQ.

As previously reported (21), all of the HLA markers for GSE are derived from two conserved or extended haplotypes, [HLA-B8, SC01, DR3] and [HLA-B44, FC31, DR7]. It is evident that for both HLA-DR3, DQ2- and HLA-DR7, DQ2-bearing haplotypes that (DR, DQ) fragments of the complete extended haplotypes were specifically enriched over (HLA-B, complotype), (complotype), or (complotype, DR, DQ) fragments in GSE. These findings support the concept that a gene in the DR, DQ region, probably a class II gene, is the susceptibility gene for GSE.

Even though MHC markers among DH patient haplotypes were very similar and the same extended haplotypes were increased as in GSE, the (SC01) fragment was specifically enriched over (B8, SC01), (SC01, DR3, DQ2), and (DR3, DQ2). Furthermore, there was only one haplotype with DR3 but not SC01, but six with SC01 but not DR3. SC01 had the highest odds ratio of any MHC marker in DH, including DQ2. All of these points support the conclusion that the DH susceptibility gene is near SC01. This was in contrast to GSE, in which DQ2 had the highest odds ratio.

The findings with respect to DH haplotypes related to [HLA-B44, FC31, DR7] were less clear cut in that both HLA-B44 and DR7 alone were often (and roughly equally) found and only one (FC31) alone occurred among patient haplotypes. Nevertheless, the presence of several instances of the (FC31, DR7, DQ2) fragment suggests that the susceptibility gene on this haplotype, too, may be between the complotypes and DR regions. Although ethnic differences between DH and GSE patients might affect the extent of fragmentation of the relevant extended haplotypes (30, 31), this would not in itself be expected to result in enrichment of some specific fragments over others.

**Table 4.** *MHC Haplotypes in Patients with GSE*

| Haplotype no. | HLA-A | HLA-B | Complotype | HLA-DR |
|---|---|---|---|---|
| Haplotypes related to (HLA-B8, SC01, DR3): | | | | |
| 38756d | 24 | 8 | FC31 | 3* |
| 40713a | 3 | 7 | SC01 | 3 |
| 35788c | 1 | 8 | SC01 | 3 |
| 37001b | 1 | 8 | SC01 | 3 |
| 38263c | 1 | 8 | SC01 | 3 |
| 38313a | 1 | 8 | SC01 | 3 |
| 38506a | 1 | 8 | SC01 | 3 |
| 38619a | 1 | 8 | SC01 | 3 |
| 38647c | 1 | 8 | SC01 | 3 |
| 39987a | 1 | 8 | SC01 | 3 |
| 40709c | 1 | 8 | SC01 | 3 |
| 40771a | 1 | 8 | SC01 | 3 |
| 42613c | 1 | 8 | SC01 | 3 |
| 42613d | 1 | 8 | SC01 | 3 |
| 44417c | 1 | 8 | SC01 | 3 |
| 11245c | 2 | 8 | SC01 | 3 |
| 11263c | 2 | 8 | SC01 | 3 |
| 38506c | 2 | 8 | SC01 | 3 |
| 40709a | 2 | 8 | SC01 | 3 |
| 40777a | 2 | 8 | SC01 | 3 |
| 43870c | 2 | 8 | SC01 | 3 |
| 38668c | 3 | 8 | SC01 | 3 |
| 38668a | 24 | 8 | SC01 | 3 |
| 35017a | 24 | 8 | SC01 | 3 |
| 37925a | 25 | 8 | SC01 | 3 |
| 11278a | 28 | 8 | SC01 | 3 |
| 11260a | 28 | 8 | SC01 | 3 |
| 34986c | 32 | 8 | SC01 | 3 |
| 38756c | 29 | 8 | SC01 | 3 |
| 38623a | 34 | 8 | SC01 | 3 |
| 35204a | 2 | 18 | F1C30 | 3‡ |
| 35788d | 1 | 57 | SC61 | 3 |
| 35811a | 11 | 39 | SC42 | 3 |
| 42136b | 2 | 44 | SC30 | 3* |
| 38553c | 3 | 7 | SC31 | 3 |
| 40713c | 2 | 44 | SC(3,905)1 | 3* |
| 42629d | 26 | 63 | S1C2(1,17) | 3 |
| Haplotypes related to (HLA-B44, FC31, DR7): | | | | |
| 42136b | 2 | 44 | SC30 | 3* |
| 40713c | 2 | 44 | SC(3,905)1 | 3* |
| 38584c | 2 | 44 | SC30 | 5 |
| 35204b | 29 | 44 | SC61 | 6 |
| 38756d | 24 | 8 | FC31 | 3* |
| 11146c | 29 | 44 | FC31 | 7 |
| 11245a | 29 | 44 | FC31 | 7 |
| 11260c | 29 | 44 | FC31 | 7 |
| 38263a | 29 | 44 | FC31 | 7 |
| 11278c | 2 | 44 | FC31 | 7 |
| 38572c | 2 | 44 | FC31 | 7 |
| 38619c | 2 | 44 | FC31 | 7 |
| 38623c | 28 | 44 | FC31 | 7 |
| 37001d | 25 | 18 | FC31 | 7 |
| 37925c | 26 | 45 | FC01 | 7 |
| 40777d | 30 | 58 | FC01 | 7 |
| 11263a | 33 | 44 | SC31 | 7 |
| 11146a | 1 | 57 | SC61 | 7‡ |
| 34986a | 30 | 13 | SC31 | 7 |
| 36051a | 2 | 13 | SC31 | 7 |
| 35811c | 24 | 22 | SC31 | 7 |
| 38553a | 29 | 14 | SC31 | 7 |
| 40771c | 2 | 62 | SC33 | 7 |
| 42629b | – | 51 | S1C3,17 | 7 |
| 44440c | 11 | 44 | SC31 | 7 |
| Other haplotypes: | | | | |
| 38584a | 3 | 7 | SC31 | 2‡ |
| 44417a | 1 | 7 | SC31 | 2‡ |
| 35017c | 2 | 60 | SB42 | 4‡ |
| 38647a | 2 | 62 | SB42 | 4‡ |
| 42136e | 3 | 60 | SC31 | 4 |
| 43870a | 26 | 35 | SC31 | 4‡ |
| 36051c | 26 | 14 | SC31 | 5 |
| 38313c | 30 | 38 | SC31 | 5 |
| 38572a | 24 | 51 | SC31 | 5 |
| 44440a | 69 | 55 | SC30 | 5 |
| 39987c | 11 | 51 | FC(3,2)0 | 6 |

* Listed twice as related to both [HLA-B8, SC01, DR3] and [HLA-B44, FC31, DR7].
‡ Extended haplotype other than those above.

Table 5. *Odds Ratios of MHC Alleles in Patients with DH and GSE with and without Removal of [HLA-B8, SC01, DR3]*

| Allele | Dermatitis herpetiformis odds ratio | | Gluten-sensitive enteropathy odds ratio | |
|---|---|---|---|---|
| | With [B8, SC01, DR3] | Without [B8, SC01, DR3] | With [B8, SC01, DR3] | Without [B8, SC01, DR3] |
| B8 | 9.8 | 3.4 | 7.5 | 0 |
| SC01 | 37.8 | 99.6 | 8.7 | 1.7 |
| DR3 | 10.6 | 5.4 | 14.7 | 11.4 |
| [B8, SC01, DR3] | 11.8 | - | 10.9 | - |
| B44 | 1.1 | 1.1 | 1.8 | 5.0 |
| FC31 | 1.3 | 2.5 | 1.8 | 0.8 |
| DR7 | 2.0 | 19.1 | 3.5 | 5.6 |
| [B44, FC31, DR7] | 2.0 | 11.4 | 4.8 | 6.4 |
| C4A*Q0 | 29.3 | 50.6 | 5.9 | 0.8 |
| DQ2 | 35.9 | 6.8 | 46.9 | 34.0 |

Odds ratios are for subjects positive for the marker.

Table 6. *Extended Haplotype Fragment Distributions in Patients with DH and GSE*

| Disease | SC01, not DR3,DQ2 no. | DR3,DQ2, not SC01 no. |
|---|---|---|
| DH | 6 | 1 |
| GSE | 0 | 8 |

$p < 0.002$.

We believe that there are a number of susceptibility genes for DH and a number for GSE at a single locus for each disease acting in an essentially, but not simply, recessive manner. Others have found that DR4 haplotypes may contribute "minor" susceptibility to GSE (32). From our results, the major susceptibility markers for GSE are DR3, DQ2 and DR7, DQ2, and the minor markers are DR4, DQ7; DR5, DQ1; DR2, DQ1; and DR6, DQ1. We cannot, however, rule out 2 distinct MHC susceptibility loci (DQ and DP related) in GSE and three (complotype, DR/DQ, and DP related) in DH. Against the latter possibility is the presence primarily of (DR3, DQ2) and (DR7, DQ2) but not (FC31) or (SC01) in GSE and SC01, rather than chiefly class II fragments in DH patient haplotypes. If both DQ and complotype genes were required for recessive expression, the minimum fragment should contain both complotype and DQ susceptibility genes.

The relatively greater diversity of HLA-A alleles on [HLA-B8, SC01, DR3] haplotypes in GSE compared with the same haplotypes in DH patients or normals, in whom 75–80% of instances carry HLA-A1, may reflect the susceptibility gene localization results. Since there was enrichment for DR, DQ fragments in GSE, one might expect less homogeneity of the HLA-A region which is $2 \times 10^6$ base pairs telomeric to the class II region. It follows that if the susceptibility locus is near the complotype region, perhaps between the latter and DR, DQ, this may result in the observed higher HLA-A1 frequency on [HLA-B8, SC01, DR3] in DH than in GSE.

Recent studies have raised the possibility of specific HLA-DP alleles being associated with GSE (33–35) and DH (36). Our finding of an increase in the frequency of a specific DP allele, DPB1*0101, in both our GSE and DH patients over normal controls supports this possibility. We did not find increases in DPB1*04 and DPB1*03 alleles in GSE, as reported by Bugawan et al. (34), nor the increase in DP3 found by Kagnoff et al. (35), but confirmed the increase in DP1 found by this group. We found DPB1*0101 to be increased, particularly in association with HLA-DR3, suggesting that the [HLA-B8, SC01, DR3] extended haplotype often extends through DP in both GSE (34) and DH (9, 36, 37) patients as well as in normals (10). In this view, the increase in the DP marker may be because of known linkage disequilibrium between [HLA-B8, SC01, DR3] and DP1 (10, 11) (DPB1*0101) but may also reflect the possibility that the DP region contains a susceptibility gene (33).

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# Isolation of a cDNA Encoding a Novel Member of the Transglutaminase Gene Family from Human Keratinocytes

## DETECTION AND IDENTIFICATION OF TRANSGLUTAMINASE GENE PRODUCTS BASED ON REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION WITH DEGENERATE PRIMERS*

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**We developed a method using a single set of degenerate oligonucleotide primers for amplification of the conserved active site of transglutaminases by reverse transcription-polymerase chain reaction (RT-PCR) and identification of the PCR products by cleavage with diagnostic restriction enzymes. We demonstrate amplification of tissue transglutaminase ($TG_C$), keratinocyte transglutaminase ($TG_K$), prostate transglutaminase ($TG_P$), the a-subunit of factor XIII, and band 4.2 protein from different human cells or tissues. Analysis of normal human keratinocytes revealed expression of a transglutaminase different from the expected and characterized transglutaminase gene products. A full-length cDNA for the novel transglutaminase ($TG_X$) was obtained by anchored PCR. The deduced amino acid sequence encoded a protein with 720 amino acids and a molecular mass of ~81 kDa. A comparison of $TG_X$ to the other members of the gene family revealed that the domain structure and the residues required for enzymatic activity and $Ca^{2+}$ binding are conserved and showed an overall sequence identity of about 35%. Two transcripts with an apparent size of 2.2 and 2.8 kilobases were detected with a specific probe for $TG_X$ on Northern blots of human foreskin keratinocyte mRNA, indicating the presence of alternatively spliced mRNAs. cDNA sequencing revealed a shorter $TG_X$ transcript lacking the sequence homologous to that encoded by exon III of other transglutaminase genes. $TG_X$ expression increased severalfold when keratinocyte cultures were induced to differentiate by suspension or growth to postconfluency, suggesting that $TG_X$ contributes to the formation of the cornified envelope.**

Transglutaminases (EC 2.3.2.13) represent a family of enzymes capable of stabilizing protein assemblies by γ-glutamyl-ε-lysine cross-links. Enzymes of this family catalyze a $Ca^{2+}$-dependent transfer reaction between the γ-carboxamide group of a peptide-bound glutamine residue and various primary amines, most commonly the ε-amino group of lysine residues (1, 2). Six different transglutaminase gene products have been characterized in vertebrates thus far by determination of their primary structure (3). In addition to the diversity on the genetic level, these enzymes have been shown to undergo a number of different posttranslational modifications such as phosphorylation, fatty acylation, and proteolytic cleavage, regulating their enzymatic activity and subcellular localization (for review, see Refs. 3, 4, 5, 6). The individual transglutaminase gene products have specialized in the cross-linking of particular proteins or tissue structures, *e.g.* factor XIIIa stabilizes the fibrin clot in hemostasis and prostate transglutaminase ($TG_P$)[1] is involved in semen coagulation (for review see Refs. 2 and 3), or have even adopted additional functions such as tissue transglutaminase ($TG_C$) in GTP-binding in receptor signaling (7, 8) or band 4.2 protein as a structural component of the cytoskeleton (9).

Three transglutaminases have been shown to be expressed in different stages of epidermal differentiation (for review, see Refs. 3, 10). Two of those, keratinocyte ($TG_K$) and epidermal ($TG_E$) transglutaminase, are associated with terminal differentiation events of keratinocytes (4, 11) and cross-link structural proteins forming the cornified cell envelope (12, 13). The third enzyme, $TG_C$ (14), is expressed in skin primarily in the basal cell layer (11, 15) and plays a role in stabilization of the dermo-epidermal junction (16–19). The importance of proper cross-linking of the cornified envelope is exemplified by the pathology seen in patients suffering from one form of the skin diseases referred to as congenital ichthyosis that has been linked to mutations in the $TG_K$ gene (20, 21).

The expression of more than one type of transglutaminase in a particular cell type, *e.g.* keratinocytes and chondrocytes, and the presence of the same gene product in different cellular compartments raises questions about the nature of the enzyme that is involved in a particular biological process, *e.g.* formation of the skin cornified envelope (4, 11) or maturation of cartilage (16, 22, 23). Sensitive and specific assays are needed to detect the transglutaminase gene products that potentially contribute to biological events. To address this issue, we have developed an assay based on PCR amplification using degenerate primers specific for the transglutaminase gene family. Analysis of human cells and tissue revealed, besides five of the known gene

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*The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF035960 and AF035961*

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[1] The abbreviations used are: $TG_P$, prostate transglutaminase; $TG_C$, tissue transglutaminase, transglutaminase type II; $TG_E$, epidermal transglutaminase, transglutaminase type III; $TG_K$, keratinocyte transglutaminase, transglutaminase type I; EGF, epidermal growth factor; KGF, keratinocyte growth factor; RT, reverse transcription; PCR, polymerase chain reaction; bp, base pair(s); aa, amino acid(s); kb, kilobase(s).

TABLE I
*Design of degenerate primers for amplification of members of the transglutaminase gene family by PCR*
Only human sequence is available for factor XIIIa. h = human, m = mouse, r = rat, I = inosine.

| Gene products (reference) | Upstream sequences and derived degenerate primer D1 | Downstream sequences and derived degenerate primer D2 |
|---|---|---|
| $hTG_C$ (14) | TATGGCCAGTGCTGGGTCTTCGCCGCCGT | TGGATGACCAGGCCGGACCTGCAGCCGGG |
| $mTG_C$ (14) | **C**********G**T**A**G** | **********A*****A******** |
| $hTG_E$ (11) | TATGGCCAGTGCTGGGTCTTTGCTGGGAC | TGGTTTGTGAGGTCTGACCTGGGCCCCCC |
| $mTG_E$ (11) | *T**********G*******A** | *****C***C**A*******A******A* |
| hB4.2 (30) | GATGGCCAGGCCTGGGTGTTGGCTGCTGT | TGGATGACGCGGCCTGCCTTGCCCCAGGG |
| mB4.2 (54) | **GAC******G*******CT******** | *******ACA*A****AT***T****A** |
| hFXIIIa (37, 38) | TATGGCCAATGCTGGGTTTTGCTGGTGT | TGGATGACAAGGCCTGACCTTCCTGTTGG |
| $hTG_K$ (31, 40) | TATGGCCAGTGCTGGGTCTTTGCTGGCGT | TGGATGAAGAGGCCGGATCTGCCCTCGGG |
| $rTG_K$ (31, 40) | *******A**********C**T** | **********A**********A** |
| $hTG_P$ (28, 42) | TTTGGCCAGTGCTGGGTGTTTGCTGGGAT | TGGATGAAGCGACCCTACGACGGCTGCAG |
| $rTG_P$ (41) | **********T**CT****AGT | ********AA***AGG*TCTACCCCAGG* |
| Consensus | TATGGCCAGTGCTGGGT_TTTGCTGG_GT | TGGATGA_GAGGCC_GACCTGC_C___GG |
| Sequence | Y G Q C W V F A G V | W M _ R P D L P _ G |
| primer | TACGGCCAATGCTGGGTITTCGCIGCAGT | CCAGGGIGAAGATCAGICCTCGCCATCCA |
| (5′ → 3′) | T G T GG | G C G T TTT |
| | C | C T |
| | T | T |

products including $TG_C$, band 4.2 protein, the a-subunit of factor XIII, $TG_K$ and $TG_P$, a novel transglutaminase gene product, $TG_X$. In the present study, we describe the full-length cDNA sequence and deduced amino acid sequence of two splice variants of this novel human gene.

## MATERIALS AND METHODS

*Reagents*—Oligonucleotides and restriction enzymes were from Oligos Etc. Inc. (Wilsonville, OR) and Promega Corp. (Madison, WI), respectively. Reagents for cell culture were from Life Technologies, Inc.

*Cells*—Human keratinocytes were isolated from neonatal human foreskin as described previously (24). Primary keratinocyte cultures were established on mitomycin C-treated mouse Swiss 3T3 fibroblast feeder layers in 3 parts Ham's F12 plus 1 part Dulbecco's modified Eagle's medium containing 2.5% fetal bovine serum, 0.4 μg/ml hydrocortisone, 8.4 ng/ml cholera toxin, 5 μg/ml insulin, 24 μg/ml adenine, 10 ng/ml epidermal growth factor (EGF; R&D Systems, Minneapolis, MN), and antibiotics (100 μg/ml streptomycin and 100 units/ml penicillin). To induce differentiation, cells were harvested by trypsinization and cultured for the indicated time in suspension in the same medium supplemented with 1.68% methylcellulose (4,000 centipoises; Fisher Scientific Corp.) (25). For experiments analyzing the effect of cell density and growth factors on differentiation, cells were grown for one passage on a feeder layer in the absence of EGF. Subsequently, cells were grown for 24 h in the absence of a feeder layer before supplementing the medium with 0.5 nM EGF, 0.5 nM keratinocyte growth factor (KGF; Promega), or 10 μl of 0.1% bovine serum albumin/ml of medium for the indicated time (25). Human dermal fibroblasts, TJ6F, were established from trypsinized foreskin tissue, and human osteosarcoma cell line MG-63 (CRL 1427) and human fibrosarcoma cell line HT1080 (CCL 121) were purchased from the American Type Culture Collection (ATCC, Rockville, MD) and cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and antibiotics. Human erythroleukemia cell line HEL was kindly provided by Dr. Mortimer Poncz, Philadelphia, PA, cultured in suspension in RPMI 1640 medium containing 12% fetal bovine serum, 1 mM pyruvate, and antibiotics, and induced to differentiate with 1.25% dimethyl sulfoxide for 2 days (26). Human platelets were collected as described (27), and a contamination with leukocytes or red blood cells was ruled out by phase contrast microscopy.

*PCR Amplification of Transglutaminases with Degenerate Primers*—Poly(A)$^+$ RNA was prepared from about $10^6$ cells or 10 μg total RNA by oligo(dT)-cellulose column chromatography using the Micro-Fast Track Kit (Invitrogen, San Diego, CA) and recovered in 20 μl of 10 mM Tris/HCl, pH 7.5. The poly(A)$^+$ RNA (5.0 μl) was reverse transcribed into DNA in a total volume of 20 μl using the cDNA Cycle Kit (Invitrogen) with either 1.0 μl of random primers (1 μg/μl) or oligo(dT) primer (0.2 μg/μl). No difference in the amount or nature of the PCR product was observed when the reverse transcription was done with random or oligo(dT) primers. cDNA from human prostate carcinoma tissue was kindly provided by Dr. Erik J. Dubbink, Rotterdam, The Netherlands (28).

PCRs were carried out with 2.5 units of *Taq* DNA polymerase (Fisher Scientific) and 25% of the reverse transcriptase reaction mixture (5.0 μl) in 100 μl of 10 mM Tris/HCl, pH 8.3, 50 mM KCl containing 2 mM $MgCl_2$, 0.2 mM dNTPs and 50 pmol of the transglutaminase-specific degenerate oligonucleotide primers D1 and D2 (see Table I). The PCR cycles were 45 s at 94 °C (denaturation), 2 min at 55 °C (annealing), and 3 min at 72 °C (elongation). A total of 37 cycles were made, with the first cycle containing an extended denaturation period (6 min) during which the polymerase was added (hot start) and the last cycle containing an extended elongation period (10 min).

The 230-bp PCR products were purified by agarose gel electrophoresis, recovered with the Wizard PCR Preps DNA Purification System (Promega), and cloned by taking advantage of the 3′ A-overhangs generated by *Taq* DNA polymerase using the Original TA-Cloning Kit (Invitrogen). Plasmid DNA was prepared with the Wizard Minipreps DNA Purification System (Promega) and sequencing performed by the dideoxy chain termination method using the Sequenase Version 2.0 Kit (U. S. Biochemical Corp.).

*Cloning of $TG_X$ by Anchored PCR*—Double-stranded cDNA was prepared from poly(A)$^+$ RNA (prepared as above) of cultured normal human keratinocytes with the Copy Kit (Invitrogen) using the oligo(dT)-*Not*I oligonucleotide (see Fig. 2) to prime first strand synthesis. $TG_X$-related sequences were amplified by anchored PCR in both directions as outlined in Fig. 2 using $TG_X$-specific oligonucleotides and additional degenerate primers (see Table II) or the oligo(dT)-*Not*I oligonucleotide for the 3′-end. The PCRs were performed under the conditions described above. Nested PCRs were done by replacing the cDNA with 1.0 μl from the first PCR reaction. Since degenerate primers to conserved sequences upstream of primer D4 did not yield PCR products, the cDNA was purified from nucleotides using the GlassMax DNA Isolation Kit (Life Technologies, Inc.) and tailed in the presence of 200 μM dCTP with 10 units of terminal deoxynucleotidyl transferase (Promega) for 30 min at 37 °C (29) to anchor the PCR at the 5′-end. The PCR reaction was anchored by performing a total of 5 cyles of one-sided PCR at a lower annealing temperature (37 °C) with the abridged anchor primer (Life Technologies, Inc.; see Fig. 2) only and was followed by transfer of 25% of the reaction at 94 °C to a new tube containing abridged anchor primer and $TG_X$-specific primer S6 (Table II) and by amplification as above. Nested PCR reactions were done with the universal amplification primer (Life Technologies, Inc.) and internal $TG_X$-specific primers (Table II) as indicated in Fig. 2.

The PCR products were gel-purified using the Geneclean II Kit (BIO 101 Inc., Vista, CA) and cloned as above. Both strands were sequenced from both directions, with additional internal primers where required, using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer Corp.) and the automated sequencing facility at the Biotechnology Center at the University of Wisconsin.

*Northern Blotting*—3 μg of poly(A)$^+$ RNA from human foreskin keratinocytes was separated in a 1.2% agarose gel containing formaldehyde and transferred to a Zeta-probe membrane (Bio-Rad). The gel was calibrated using the 0.24–9.5-kb RNA ladder (Life Technologies, Inc.). For preparation of the probes, an ~700-bp DNA fragment encoding the 3′-end of $TG_X$, $TG_C$, band 4.2, or $TG_K$ was prepared by restriction with *Pst*I and *Acc*I, *Stu*I and *Bsu*36I, *Xho*I, or *Xcm*I and *Xho*I, respectively. cDNAs encoding human $TG_C$, band 4.2 protein, and $TG_K$ were kindly

provided by Drs. Peter J. A. Davies, Houston, TX (14), Carl M. Cohen, Boston, MA (30), and Robert H. Rice, Davis, CA (31), respectively. $^{32}$P-labeled probes were prepared using random prime labeling (Multiprime DNA labeling system; Amersham Int., Amersham, UK). The membrane was hybridized with the probe at 42 °C overnight, washed with a final stringency of 0.1 × SSC, 1% SDS at 65 °C for 30 min, and exposed to x-ray film (Kodak, Rochester, NY) for the indicated time period.

*Amplification of $TG_X$ from Different Cells*—cDNA was prepared as described above and a 225-bp fragment of $TG_X$ was amplified from 1.0 μl of cDNA with specfic primers S4 and S9 (Table II) using the PCR conditions described above except for annealing at 60 °C.

## RESULTS AND DISCUSSION

*Design of PCR for Amplification of Transglutaminase Gene Products*—To analyze the expression of transglutaminases when starting material is limited, we undertook an effort to design primers capable of specifically amplifying transglutaminase sequences by PCR. Alignment and comparison of the different known transglutaminase gene products on the nucleotide level revealed several conserved regions, particularly in the catalytic core domain (Table I; see also Fig. 7), that could serve as targets for primers. A single set of degenerate oligonucleotide primers (Table I) that amplify by PCR a 230-bp DNA fragment encoding the highly conserved active site region of transglutaminases (Fig. 1) was identified by screening of oligonucleotides based on different conserved regions in PCR reactions using plasmid DNA of different transglutaminases. The primers are based on the sequence YGQCWVFAGV (see Fig. 7, aa 274–283 in $TG_X$), which includes the active site cysteine residue, and WM_RPDLP_G (aa 342–351) (Table I). Initial attempts with shorter oligonucleotides (18 bp) designed after the conserved sequences LFNPWC (see Fig. 7, aa 138–143 in $TG_X$), QCWVFA (aa 276–281), and WNFHVW (aa 333–338) were unsuccessful. Also, degenerate oligonucleotides based on the sequence WQ_LDATPQE (see Fig. 7, aa 355–364 in $TG_X$) and F_LLFNPWC (aa 135–143) did not yield PCR products.

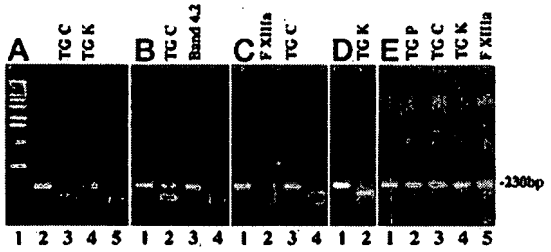


FIG. 1. **Amplification of transglutaminases from different human cell lines or tissues.** A 230-bp fragment corresponding to the active site of transglutaminases was amplified with degenerate primers D1 and D2 (Table I) by RT-PCR from MG-63 osteosarcoma cells (*lane A2*), HEL erythroleukemia cells (*lane B1*), platelets (*lane C1*), keratinocytes (*lane D1*), and prostate carcinoma tissue (*lane E1*). Cleavage of the PCR products with restriction enzymes revealed the type of transglutaminase expressed: *Sca*I, $TG_C$; *Bst*EII, band 4.2 protein; *Eco*RI, factor XIII a-subunit; *Bsp*1286I, $TG_K$; and *Tth*111I, $TG_P$. In osteosarcoma cells, *Sca*I (*lane A3*), *Bsp*1286I (*lane A4*), and *Sca*I + *Bsp*1286I (*lane A5*) reveal $TG_C$ and $TG_K$; in erythroleukemia cells, *Sca*I (*lane B2*), *Bst*EII (*lane B3*), and *Sca*I + *Bst*EII (*lane B4*) reveal $TG_C$ and band 4.2 protein; in platelets, *Eco*RI (*lane C2*), *Sca*I (*lane C3*), and *Eco*RI + *Sca*I (*lane C4*) reveal the a-subunit of factor XIII and $TG_C$; in keratinocytes, *Bsp*1286I (*lane D2*) reveals $TG_K$; and in prostate carcinoma tissue, *Tth*111I (*lane E2*), *Sca*I (*lane E3*), *Bsp*1286I (*lane E4*), and *Eco*RI (*lane E5*) reveal $TG_P$, $TG_C$, $TG_K$, and the a-subunit of factor XIII. DNA fragments were analyzed by electrophoresis in 1% agarose gels calibrated with the 1-kb DNA ladder (*lane A1*; Life Technologies, Inc.).

*Identification of Transglutaminase Gene Products by Restriction Analysis*—A facile method to identify the nature of the PCR products amplified with the degenerate primers is restriction analysis. Restriction sites conserved among species for a particular transglutaminase gene but not present in PCR products derived from other members of the gene family allow identification. Based on a comparison of the sequence information of transglutaminases for man, the following restriction enzymes gave cleavage patterns diagnostic of the six different human gene products: *Sca*I for $TG_C$, *Bcl*I and *Nco*I (*Ava*I) for $TG_E$, *Bst*EII for band 4.2 protein, *Eco*RI for factor XIII a-subunit, *Bsp*1286I and *Nco*I for $TG_K$, and *Tth*111I for $TG_P$ (Fig. 1). This selection of restriction enzymes would also work with known rat or mouse sequences.

*Amplification of Transglutaminase Gene Products from Human Cells or Tissue*—We selected different human cells or tissues that are known to express a distinct transglutaminase gene product to test whether we could amplify all gene products of the transglutaminase family. Sequence information for all six characterized genes is only available in man.

$TG_C$ is expressed in many cell types and tissues in the vertebrate body (14, 17, 22, 32), and we selected primary dermal fibroblasts (33) and two tumor cell lines, fibrosarcoma HT1080 and osteosarcoma MG-63 (34), for our analysis. In fibroblasts and HT1080 fibrosarcoma cells, only $TG_C$ was de-

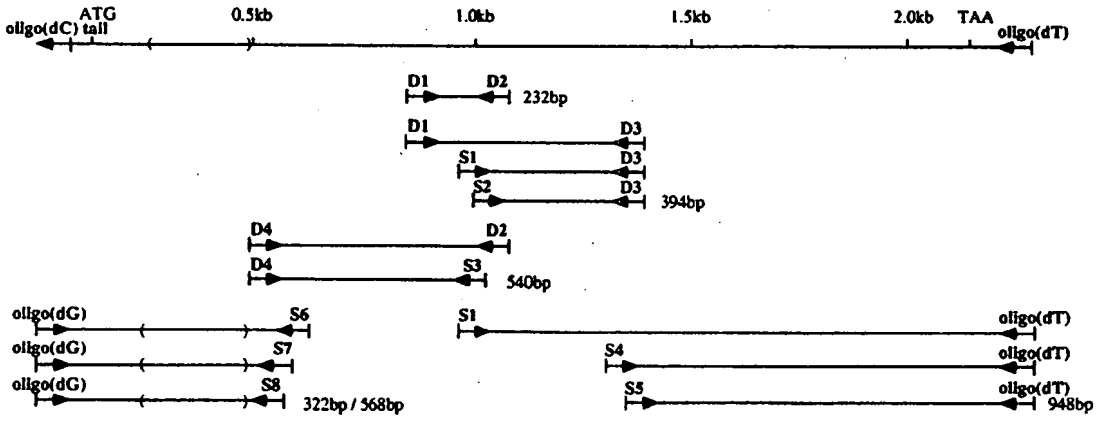


FIG. 2. **PCR strategy for amplification of cDNA sequences of $TG_X$.** The *top line* represents the cDNA for $TG_X$ with the start and stop codon indicated. *Brackets* indicate the alternatively spliced sequence. *Below* is an outline of the PCR strategy, showing the consecutive PCR reactions performed with nested oligonucleotide primers to obtain PCR products visible in ethidium bromide-stained agarose gels. The length of the final PCR products is given on the *right*. The sequences of the oligonucleotide primers are given in Tables I and II. The oligo(dT)-*Not*I unidirectional primer (Invitrogen), 5′-AACCCGGCTCGAGCGGCCGCT$_{(18)}$, was used as the 3′-anchoring primer. The abridged anchor primer (Life Technologies, Inc.), 5′-GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG, was used as the 5′-anchoring primer. In this case, the subsequently used primer for nested PCR was a shortened oligonucleotide, universal amplification primer (Life Technologies, Inc.) consisting of the first 20 nucleotides of the abridged anchor primer.

TABLE II
*Sequences of oligonucleotide primers used for PCR of $TG_X$*

Primers are numbered and were used for amplification of $TG_X$-specific sequences as indicated in Fig. 2. "D" indicates degenerate primer; "S" indicates $TG_X$-specific primer. Forward primers (sense) are labeled "f"; reverse primers (antisense) are labeled "r". The sequence position of the primers is based on the sequence given in Fig. 3*A*. The following abbreviations are used for degenerate positions in oligonucleotides: M = A, C; R = A, G; S = C, G; W = A, T; Y = C, T; I = inosine.

| Designation | Sequence | Orientation | Position |
|---|---|---|---|
| D3 | 5′-CTCTCYTCIICISWICCYTCTGGGWAYTTGTA | r | 1092–1123 |
| D4 | 5′-TGGAIIAIGARGAIGAGMGRSARGARTATGT | f | 214–244 |
| S1 | 5′-TAGATGAGTATTATGACAACACAGGCAGG | f | 703–731 |
| S2 | 5′-AGGATTTTGGGGAATAAGAAGAAGGATAC | f | 729–757 |
| S3 | 5′-TCCTTCTTCTTATTCCCCAAAATCCTGCC | r | 726–754 |
| S4 | 5′-TTCACCAGGACACGAGTTCTGTTGGCA | f | 1009–1035 |
| S5 | 5′-CAAAGAGCATCCAGAGTGACGAGCGGG | f | 1048–1074 |
| S6 | 5′-TCTGTGGCTGGGTCAGTCTGGAAGTGC | r | 368–394 |
| S7 | 5′-TGTCIATAGTTICAGGGAIATGGGCGG | r | 290–316 |
| S8 | 5′-CAGTTCTTGCTGCCTTGGTAGATGAAGCC | r | 258–286 |
| S9 | 5′-GGGCTGTCCTGGCTCAGTGATGTGGGC | r | 1208–1234 |

tectable (results not shown), wheras MG-63 osteosarcoma cells expressed $TG_C$ and $TG_K$ (Fig. 1*A*). Band 4.2 protein is a membrane cytoskeleton component expressed at a high level in erythroid cells (30, 35). For this reason, a human erythroleukemia cell line (HEL) was tested. Erythrocytes are also known to express significant amounts of $TG_C$ (2, 36). We detected both $TG_C$ and band 4.2 protein in HEL cells (Fig. 1*B*). Platelets were chosen for amplification of the a-subunit of factor XIII because they are the major source for factor XIII a-subunit in plasma (37, 38, 39) and have been shown to contain mRNA even though they are devoid of a nucleus (27). The amplification showed that the a-subunit of factor XIII is the predominant transcript in platelets, but $TG_C$ was also detected (Fig. 1*C*). $TG_K$ and $TG_E$ contribute to the formation of the cornified envelope in skin in distinct steps of keratinocyte differentiation (4, 11, 20, 40). Therefore, primary keratinocyte cultures that were induced to differentiate by culture in suspension were analyzed. $TG_K$ was detected in adherent cells (Fig. 1*D*) as well as in nonadherent cells (result not shown). We were unable to detect $TG_E$ after culture in suspension for up to 24 h. The inability to detect $TG_E$ may be due to the fact that the sequence of $TG_E$ differs more from the consensus used to design the primers than other transglutaminase sequences (Table I). On the other hand, the expression of $TG_E$ in human epidermis has been found to be very low and not detectable in cultured human keratinocytes (11). $TG_P$ is an androgen-regulated protein involved in semen coagulation, and its expression is restricted to prostate (28, 41, 42). Since no human cell line with known $TG_P$ expression was available, human prostate tissue was tested. $TG_P$ was the major transcript deteced in prostate carcinoma tissue, but several other transglutaminases, $TG_C$, the a-subunit of factor XIII, and $TG_K$ were present as well, which is to be expected in a vascularized tissue sample that is composed of many different cell types.

To confirm the identity of the PCR products, the 230-bp DNA fragments were cloned using the A-overhangs produced by *Taq* DNA polymerase and sequenced. To facilitate cloning of rare PCR products, portions of the DNA were cleaved by a restriction enzyme that degrades a known PCR product, and the remainder was cloned as above. Clones containing sequences of a predicted type of transglutaminase were obtained in all cases, demonstrating that the assay is reliable. Keratinocytes contained a minor amount of PCR products different from $TG_K$, which we were unable to identify by restriction analysis (see Fig. 1*D*, *lane 2*). Cloning and analysis of the clones derived from these products revealed that $TG_C$ was expressed in adherent keratinocytes, as has been suggested previously (Refs. 11 and 15; see also Fig. 4). Unexpectedly, we also found a transcript for a transglutaminase different from the previously characterized human transglutaminase genes. We designate this novel transglutaminase in the following as $TG_X$ since its function is at present unknown.

*Cloning of $TG_X$ from Human Keratinocytes by Anchored PCR and Its Deduced Amino Acid Sequence*—To obtain further sequence information on $TG_X$, oligo(dT)-primed double-stranded cDNA was prepared from poly(A)$^+$ RNA from primary keratinocytes isolated from human foreskin. The strategy of the anchored PCR is summarized in Fig. 2, and the sequence of the oligonucleotide primers is given in Table II. To exclude sequence mutations introduced by *Taq* DNA polymerase, all DNA fragments were amplified at least twice in independent reactions, and the sequences of several cloned PCR products were determined and compared. Briefly, sequences of the 3′-end of $TG_X$ were amplified by consecutive PCR reactions using degenerate primer D1 and $TG_X$-specific primers S1 and S2 together with degenerate primer D3, which is derived from the conserved amino acid sequence YKYPEGS_EER (Fig. 7, aa 443–453 in $TG_X$). The residual 3′-sequence was amplified by sequential PCR reactions using $TG_X$-specific primers S1, S4, and S5 in combination with the oligo(dT)-*Not*I primer used for cDNA priming. Sequences 5′ of the active site were amplified in consecutive PCR reactions using degenerate primer D2 and $TG_X$-specific primer S3 together with degenerate oligonucleotide D4, which is based on an upstream cluster of conserved amino acids, *i.e.* LD_E_ER_EYV (Fig. 7, aa 150–160 in $TG_X$). Attempts to amplify sequences upstream of primer D4 with additional degenerate oligonucleotides failed. To obtain more information on the 5′-end of $TG_X$, we used a 5′-rapid amplification of cDNA ends approach (43). A poly(dC) tail was added to the cDNA using terminal deoxynucleotidyl transferase to anchor the PCR reaction with an oligo(dG) primer (abridged anchor primer). The reaction was anchored with the abridged anchor primer at low annealing temperature, and a first round of amplification was performed with abridged anchor primer and $TG_X$-specific primer S6. Subsequent reactions with nested primers, universal amplification primer and $TG_X$-specific primers S7 and S8, yielded $TG_X$-related PCR products (Fig. 2). However, heterogeneity of the sequence upstream of primer D4 was encountered, causing considerable difficulties in obtaining 5′-sequence. Three different sequences have been obtained 5′ of the sequence EDAVY (Fig. 7, aa 145–149 in $TG_X$), two of which have been fully characterized and are described in the following. The third version deviates at the same nucleotide position but yields a stop codon in the reading frame 63 nucleotides 5′ of the presumptive splice point, indicating that the third variant arose from failure of proper splicing out of an intron. An understanding of the significance of this product consequently awaits information on the gene structure.

A

```
                                                  M  A  Q  G  L  E      6
human                                 CAGCTACCATGGCCCAAGGGCTAGAA     26

                  V  A  L  T  D  L  Q  S  S  R  N  N  V  R  H  H  T  E  E  I     26
human     GTGGCCCTCACAGACCTCCAGAGCTCCAGAAATAATGTGCGGCACCACACGGAGGAGATC     86

                  T  V  D  H  L  L  V  R  R  G  Q  A  F  N  L  T  L  Y  F  R     46
human     ACTGTGGACCACCTGCTTGTTCGCCGGGGCCAGGCCTTCAACCTCACCCTGTACTTCAGG    146

                  N  R  S  F  Q  P  G  L  D  N  I  I  F  V  V  E  T  .  E  D     65
human     AACCGGAGCTTCCAGCCAGGCCTGGACAACATCATCTTCGTGGTTGAAACT...GAGGAT    203

                  A  V  Y  L  D  S  E  P  Q  R  Q  E  Y  V  M  N  D  Y  G  F     85
human     GCTGTCTACTTGGACAGTGAACCCCAGAGGCAGGAGTATGTCATGAATGATTATGGCTTC    263

                  I  Y  Q  G  S  K  N  W  I  R  P  C  P  W  N  Y  G  Q  F  E    105
human     ATCTACCAAGGCAGCAAGAACTGGATCCGCCCATGTCCCTGGAACTATGGACAGTTTGAA    323

                  D  K  I  I  D  I  C  L  K  L  L  D  K  S  L  H  F  Q  T  D    125
human     GACAAAATCATAGACATCTGCCTGAAGCTGCTAGACAAGAGCCTGCACTTCCAGACTGAC    383

                  P  A  T  D  C  A  L  R  G  S  P  V  Y  V  S  R  V  V  C  A    145
human     CCAGCCACAGACTGTGCTCTGCGGGGAAGCCCCGTCTACGTCAGCAGAGTGGTGTGTGCC    443

                  M  I  N  S  N  D  D  N  G  V  L  N  G  N  W  S  E  N  Y  T    165
human     ATGATCAACAGCAATGATGATAATGGGGTGCTCAATGGAAACTGGAGTGAGAATTACACA    503

                  D  G  A  N  P  A  E  W  T  G  S  V  A  I  L  K  Q  W  N  A    185
human     GACGGCGCCAACCCTGCGGAGTGGACGGGCAGCGTGGCCATCCTGAAGCAGTGGAACGCC    563

                  T  G  C  Q  P  V  R  Y  G  Q  C  W  V  F  A  A  V  M  C  T    205
human     ACAGGCTGCCAGCCCGTGCGCTACGGGCAATGCTGGGTCTTTGCTGCCGTCATGTGCACA    623

                  V  M  R  C  L  G  I  P  T  R  V  I  T  N  F  D  S  G  H  D    225
human     GTGATGAGGTGTCTGGGGATCCCTACCCGTGTGATCACCAACTTCGACTCTGGCCACGAT    683

                  T  D  G  N  L  I  I  D  E  Y  Y  D  N  T  G  R  I  L  G  N    245
human     ACAGATGGAAACCTGATCATAGATGAGTATTATGACAACACAGGCAGGATTTTGGGGAAT    743

                  K  K  K  D  T  I  W  N  F  H  V  W  N  E  C  W  M  A  R  K    265
human     AAGAAGAAGGATACTATCTGGAACTTCCATGTCTGGAATGAGTGCTGGATGGCCCGGAAG    803

                  D  L  P  P  A  Y  G  G  W  Q  V  L  D  A  T  P  Q  E  M  S    285
human     GATCTGCCCCCTGCATATGGAGGCTGGCAGGTGCTGGACGCCACACCTCAGGAGATGAGC    863

                  N  G  V  Y  C  C  G  P  A  S  V  R  A  I  K  E  G  E  V  D    305
human     AACGGCGTCTACTGCTGTGGCCCTGCCTCTGTCAGAGCCATCAAAGAAGGAGAAGTGGAC    923

                  L  N  Y  D  T  P  F  V  F  S  M  V  N  A  D  C  M  S  W  L    325
human     CTGAACTATGACACGCCCTTTGTGTTTTCGATGGTGAATGCTGACTGCATGTCCTGGCTC    983

                  V  Q  G  G  K  E  Q  K  L  H  Q  D  T  S  S  V  G  N  F  I    345
human     GTCCAGGGAGGGAAGGAGCAGAAGCTTCACCAGGACACGAGTTCTGTTGGCAATTTTATC   1043

                  S  T  K  S  I  Q  S  D  E  R  D  D  I  T  E  N  Y  K  Y  E    365
human     AGCACAAAGAGCATCCAGAGTGACGAGCGGGATGACATCACAGAGAACTACAAGTATGAA   1103

                  E  G  S  L  Q  E  R  Q  V  F  L  K  A  L  Q  K  L  K  A  R    385
human     GAAGGATCCCTCCAGGAGAGGCAGGTGTTTCTGAAGGCTCTGCAGAAGCTGAAGGCTAGA   1163

                  S  F  H  G  S  Q  R  G  A  E  L  Q  P  S  R  P  T  S  L  S    405
human     AGCTTCCATGGCTCCCAAAGAGGAGCAGAGTTGCAACCTTCCAGGCCCACATCACTGAGC   1223

                  Q  D  S  P  R  S  L  H  T  P  S  L  R  P  S  D  V  V  Q  V    425
human     CAGGACAGCCCTCGGAGCCTGCATACACCTTCCCTTCGACCCAGTGATGTGGTGCAAGTC   1283

                  S  L  K  F  K  L  L  D  P  P  N  M  G  Q  D  I  C  F  V  L    445
human     TCCCTGAAATTCAAGCTGCTCGACCCGCCCAACATGGGCCAGGATATATGCTTTGTCCTG   1343

                  L  A  L  N  M  S  S  Q  F  K  D  L  K  V  N  L  S  A  Q  S    465
human     CTGGCCCTCAACATGTCCTCCCAGTTCAAGGACCTCAAAGTGAACCTGAGTGCCCAGTCT   1403

                  L  L  H  D  G  S  P  L  S  P  F  W  Q  D  T  A  F  I  T  L    485
human     CTGCTGCACGATGGCAGCCCCCTGTCCCCATTCTGGCAGGACACAGCGTTCATCACACTC   1463

                  S  P  K  E  A  K  T  Y  P  C  K  I  S  Y  S  Q  Y  S  Q  Y    505
human     TCTCCTAAAGAAGCAAAGACCTACCCCTGCAAAATCTCCTATTCCCAGTACAGCCAGTAC   1523

                  L  S  T  D  K  L  I  R  I  S  A  L  G  E  E  K  S  S  P  E    525
human     CTGTCAACAGACAAGCTGATCCGCATCAGTGCCCTGGGTGAAGAGAAAAGCAGTCCTGAG   1583

                  K  I  L  V  N  K  I  I  T  L  S  Y  P  S  I  T  I  N  V  L    545
human     AAAATCCTGGTGAACAAGATCATCACCTTATCTTATCCAAGCATCACGATTAATGTTCTA   1643

                  G  A  A  V  V  N  Q  P  L  S  I  Q  V  I  P  S  N  P  L  S    565
human     GGAGCAGCCGTTGTGAACCAGCCACTCTCCATACAGGTGATATTTTCAAACCCCCTCTCG   1703

                  E  Q  V  E  D  C  V  L  T  V  E  G  S  G  L  F  K  K  Q  Q    585
human     GAGCAGGTTGAGGACTGTGTGCTGACTGTGGAAGGAAGTGGCCTCTTCAAGAAACAGCAG   1763

                  K  V  F  L  G  V  L  K  P  Q  H  Q  A  S  I  I  L  E  T  V    605
human     AAAGTCTTCCTTGGAGTCCTCAAACCCCAACACCAAGCAAGCATCATTCTGGAGACCGTC   1823
```

FIG. 3. **Nucleotide sequence and deduced amino acid sequence of human $TG_X$.** The full-length sequence of the short version of $TG_X$ is shown (*A*) with dots marking the position of the 82 amino acid insert (*B*) in the long version. The initiation and termination codons are *underlined*.

FIG. 3—*continued*

```
                P  F  K  S  G  Q  R  Q  I  Q  A  N  M  R  S  N  K  F  K  D   625
human   CCCTTCAAGAGTGGACAAAGGCAGATCCAAGCTAATATGAGAAGCAACAAGTTTAAGGAC  1883

                I  K  G  Y  R  N  V  Y  V  D  F  A  L  och                  638
human   ATTAAGGGTTACAGGAATGTTTATGTAGACTTTGCATTATAATTCTGGAACAACGCGCC  1943

human   AGACGTGTGAGTTTC                                                 1958

B               .  G  P  L  S  D  L  A  L  G  T  R  A  V  F  S  L  A  R  H    19
human   ...GGACCGCTGTCAGACCTGGCCTTGGGGACTCGGGCTGTGTTCAGCCTGGCACGCCAT    57

                H  S  P  S  P  W  I  A  W  L  E  T  N  G  A  T  S  T  E  V    39
human   CACAGCCCCAGCCCCTGGATTGCCTGGCTGGAGACCAATGGGGCCACCTCCACAGAGGTG    117

                S  L  C  A  P  P  T  A  A  V  G  R  Y  L  L  K  I  H  I  D    59
human   AGCTTGTGCGCTCCTCCCACGGCGGCCGTGGGTCGGTACCTCTTGAAAATCCACATCGAC    177

                S  F  Q  G  S  V  T  A  Y  Q  L  G  E  F  I  L  L  P  N  P    79
human   TCCTTCCAGGGGTCTGTGACGGCCTACCAGCTAGGGGAGTTCATCCTGCTTTTCAATCCC    237

                W  C  P  .                                                   82
human   TGGTGCCCA...                                                       246
```

The obtained sequence information consists of 1958 nucleotides containing an open reading frame of 1914 bp for the short form of $TG_X$ and of 2204 nucleotides with an open reading frame of 2160 bp for the long form of $TG_X$, respectively (Fig. 3, *A* and *B*). The probable initiation codon is present in the sequence ACCATGG that conforms to the consensus identified by Kozak (44) as a signal for efficient translation in higher eukaryotes. No polyadenylation signal (AATAAA) was recognized in the short 3′-untranslated region following the termination codon (TAA), indicating that it might be incomplete. However, repeated synthesis of double-stranded cDNA and PCR with different primers under various conditions did not yield additional 3′-sequence. All isolated cDNAs end within 9–34 nucleotides downstream of the pentanucleotide ATAAA at position 1922, *i.e.* at position 1935, 1938, 1939, 1942, 1943, and 1958. This pentanucleotide has been shown to function as a polyadenylation signal in other genes (45) and might be functional in $TG_X$, giving rise to a very short 3′-untranslated region. The deduced protein for the short form of $TG_X$ consists of 638 amino acids and has a calculated molecular mass of 71,915 Da and an isoelectric point of 5.9. The deduced protein for the long form of $TG_X$ consists of 720 amino acids and has a calculated molecular mass of 80,764 Da and an isoelectric point of 6.0.

*Expression of the Novel $TG_X$ and Other Transglutaminase Genes in Human Keratinocytes*—cDNA probes spanning the sequence that encodes the two C-terminal barrel domains of different human transglutaminases were used to detect the novel $TG_X$ and other transglutaminase gene products known to be expressed in keratinocytes on a Northern blot of human foreskin mRNA (Fig. 4). mRNAs of the expected sizes were detected for $TG_C$, 3.7 kb, and $TG_K$, 2.7 kb (14, 40). Two different mRNAs with sizes of about 2.2 and 2.8 kb were detected for $TG_X$, indicating that alternative processing of the transcript for $TG_X$ occurs. A previously described approximately 2.4-kb band detected with a degenerate oligonucleotide on a Northern blot of human foreskin that was assumed to be band 4.2 protein, based on its size (11), is likely to be identical to the smaller transcript of $TG_X$. This is further supported by the fact that we were unable to detect transcripts of band 4.2 protein with a specific probe (results not shown). The probes used displayed no significant cross-hybridization as indicated by the distinct migration of the detected mRNAs for the different gene products in the gel. The relative abundance of the transcripts for $TG_X$:$TG_K$:$TG_C$ is about 3:80:1. This corresponds well with the results from the PCR amplification of transglutaminases using the degenerate primers D1 and D2 (see Fig. 1*D*).

The cDNA sequence of the short form of $TG_X$ is identical to the sequence of the long form with the exception that it lacks the sequence encoded by exon III in other transglutaminase genes (Table III). The sizes of the mRNAs of $TG_X$ are larger

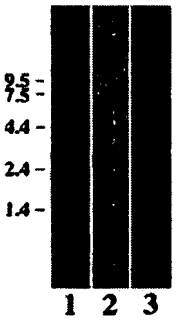


FIG. 4. **Size of transcripts of $TG_X$ in human keratinocytes.** Northern blot containing 3 μg of poly $(A)^+$ RNA of adherent keratinocytes probed consecutively with a ~700-bp fragment comprising the two C-terminal β-barrel domains of $TG_X$ (*lane 1*), $TG_K$ (*lane 2*), and $TG_C$ (*lane 3*). The blot was exposed for 3 days ($TG_X$, *lane 1*), 4 h ($TG_K$, *lane 2*), and 4 days ($TG_C$, *lane 3*). The migration position of RNA size markers is indicated on the *left*.

than expected from sequencing data. This is most likely due to the presence of additional 5′ or 3′ non-coding sequences. The smaller, more abundant mRNA might result from alternative splicing of the sequence encoded by exon III. Alternatively spliced mRNAs have been described for $TG_C$ (46, 47), band 4.2 protein (9, 48, 49), and $TG_P$.[2] No common pattern for alternative splicing is evident from the current data, and different exons are apparently alternatively processed in the different gene products. However, a band 4.2 isoform lacking exon III has been found in endothelial cells (9), and a putative $TG_P$ isoform lacks part of exon III.[2]

To analyze the expression of $TG_X$ in relation to terminal differentiation of keratinocytes, normal human keratinocytes were induced to differentiate by culture in suspension in a semi-solid methylcellulose medium. $TG_X$ was amplified by RT-PCR from an identical amount of RNA using $TG_X$-specific primers (Fig. 5). Even though $TG_X$ was present in adherent cells, it appeared to be induced in differentiating cells (Fig. 5, *lanes 7* and *8*). To corroborate this result, the expression of $TG_X$ was analyzed by semi-quantitative PCR in preconfluent and postconfluent keratinocyte cultures in the presence or absence of either EGF or KGF (Fig. 6). EGF is well known to support keratinocyte growth while KGF has recently been shown to attenuate differentiation specifically in postconfluent cultures (25). A severalfold increase in $TG_X$ expression was associated with cell density-induced differentiation (Fig. 6*B*, compare *lanes 1-3* with *4-6*). Both, EGF- and KGF-treated keratinocytes exhibited decreased levels of $TG_X$ expression relative to

[2] Thelen, K., Zippelius, A., Oberneder, R., Rietmueller, G., and Pantel, K., GenBank™/EBI Data Bank accession number U79008.

TABLE III

*Comparison of splice donor and acceptor sites used in different transglutaminases*

The splice donor and acceptor sites for the short and long form of $TG_X$ are based on the cDNA sequences and are represented in alignment with known splice sites in other transglutaminase genes. Residues consistent with the splice site consensus sequence (MAG/GTRAG and YAG/G) are underlined.

| Gene product (reference) | Donor sequence | Exons | Acceptor sequence |
|---|---|---|---|
| $hTG_X$ long | W C P<br>TGGTGCCCAG | III/IV? | E D A V<br>AGGATGCTGTC |
| $hTG_X$ short | V E T<br>GTTGAAACTG | II/IV? | E D A V<br>AGGATGCTGTC |
| $hTG_C$ (55) | W C P<br>TGGTGCCCAGgtgagccaca | III/IV | A D A V<br>CGGATGCTGTG |
| $hTG_E$ (56) | W L N<br>TGGCTGAATGgtaggtgtct | III/IV | V D S V<br>tatcaaatagTGGATAGCGTC |
| hB4.2 (48) | W N R<br>TGGAATAGAGgtaagtttga | III/IV | E D A V<br>ctctcaccagAGGATGCTGTG |

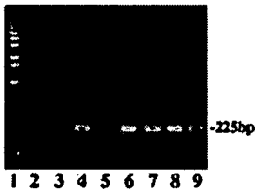


FIG. 5. **Amplification of the novel transglutaminase $TG_X$ from different human cell lines.** A 225-bp fragment of $TG_X$ was amplified by RT-PCR using specific primers S4 and S9 (Table II) from dermal fibroblasts (*lane 2*), HT1080 fibrosarcoma cells (*lane 3*), MG-63 osteosarcoma cells (*lane 4*), platelets (*lane 5*), HEL erythroleukemia cells (*lane 6*), adherent (*lane 7*) and non-adherent (*lane 8*) keratinocytes, and a fetal human skin cDNA library (*lane 9*; 18 weeks gestation, Invitrogen). Normal human keratinocytes were analyzed either prior to (*lane 7*) or after culture in suspension for 4 h (*lane 8*). PCR products were analyzed by electrophoresis in 1% agarose gels calibrated with the 1-kb DNA ladder (*lane 1*; Life Technologies, Inc.).

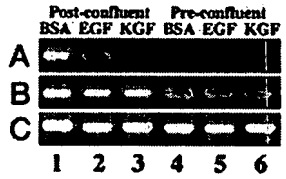


FIG. 6. **Expression of $TG_X$ in differentiating keratinocytes.** Normal human keratinocytes were treated with bovine serum albumin (*lanes 1* and *4*), 0.5 nM EGF (*lanes 2* and *5*), or 0.5 nM KGF (*lanes 3* and *6*) in standard medium for 3 days (preconfluent, *lanes 4–6*) or 10 days (postconfluent, *lanes 1–3*). *Panel A* shows amplification of transglutaminases by RT-PCR with degenerate primers D1 and D2; *panel B* shows amplification of $TG_X$ with specific primers S4 and S9. Amplification of glyceraldehyde 3-phosphate dehydrogenase with a control primer set (600-bp fragment; Stratagene) shows that equal amounts of message are present in the different samples (*panel C*). All primer sets span intron-exon boundaries, thereby ensuring that the PCR products are derived from mRNA. PCR products were analyzed in 1% agarose gels.

the control in preconfluent cultures (Fig. 6*B*, compare *lanes 4–6*). In postconfluent keratinocyte cultures, $TG_X$ expression is not significantly altered by EGF or KGF (Fig. 6*B*, *lanes 1–3*). However, amplification of transglutaminases with the degenerate oligonucleotides revealed a pattern of expression that is consistent with the pattern of transglutaminase activity measured in these cultures (results not shown; see Ref. 25) and is likely to reflect largely the expression of $TG_K$ that is the predominant type of enzyme expressed (see Fig. 1*D*).

*Structural Features of $TG_X$*—A comparison of $TG_X$ with the previously characterized human transglutaminases reveals that the structural requirements for transglutaminase activity and $Ca^{2+}$ binding are conserved (Fig. 7). The overall sequence identity between $TG_X$ and $TG_C$, $TG_E$, band 4.2 protein, the a-subunit of factor XIII, $TG_K$, or $TG_P$ is 40.1, 42.3, 31.6, 32.7, 34.9, and 31.0%, respectively. A closer comparison shows that $TG_X$ is more closely related to the evolutionary lineage including $TG_C$, $TG_E$, and band 4.2 protein (see Ref. 3) than the other transglutaminases (Table IV). The catalytic mechanism of transglutaminases has been solved based on biochemical data available for several transglutaminases (for review, see Refs. 1 and 2) and the x-ray crystallographic structure of the factor XIII a-subunit dimer (50). The reaction center is formed by the core domain and involves hydrogen-bonding of the active site Cys to a His and Asp residue to form a catalytic triad reminiscent of the Cys-His-Asn triad found in the papain family of cysteine proteases (51). The residues comprising the catalytic triad are conserved in $TG_X$ ($Cys^{277}$, $His^{336}$, $Asp^{359}$) (Fig. 7), and the core domain shows a high level of conservation as indicated by a sequence identity of about 50% between $TG_X$ and the other transglutaminases (Table IV). A Tyr residue in barrel 1 domain of the a-subunit of factor XIII is hydrogen-bonded to the active site Cys residue, and it has been suggested that the glutamine substrate attacks from the direction of this bond to initiate the reaction based on analogy to the cysteine proteases (52). In $TG_X$, the Tyr residue has been replaced by $His^{549}$ (Fig. 7), which is expected to be a conservative change. Another set of hydrogen bonds in the a-subunit of factor XIII involving residues $His^{342}$-$Glu^{434}$ and $Asp^{343}$-$Arg^{11'}$ (located in the activation peptide of the second subunit in the dimer), which have been suggested to guide the lysine substrate to the active site (50), are not conserved in that form in $TG_X$ (Fig. 7). Crystallization experiments with factor XIIIa indicated that four residues are involved in binding of a $Ca^{2+}$ ion, including the main chain carbonyl of $Ala^{457}$ and the side chain carboxyl groups of $Asp^{438}$, $Glu^{485}$, and $Glu^{490}$ (52). All three acidic residues are conserved in $TG_X$ (Fig. 7). A unique insertion of about 30 amino acids is present between the catalytic core domain and the *C*-terminal barrel domains in $TG_X$ (Fig. 7). A smaller insertion of about 10 amino acids was found in $TG_E$, and $TG_E$ has been shown to require activation by a conformational change occurring upon proteolytic cleavage in this flexible connecting loop (11). Cleavage between these domains has also been observed in $TG_K$ and in the a-subunit of factor XIII (Fig. 7). While the cleaved form of $TG_K$ is highly active (4), contradictory results have been reported with regard to the activity of factor XIIIa that has been cleaved by thrombin at this site (38, 53). Proteolytic activation of transglutaminases, probably by a member of the calpain family, seems to be a common feature for the enzymes involved in epidermal differentiation (4), and the extended flexible hinge region between the core domain and the *C*-terminal barrel domains in $TG_X$ should be prone to proteolytic attack.

Based on the similarity of $TG_X$ to the other active members of the transglutaminase protein family, it is tempting to speculate that the characterized cDNA is encoding an active transglutaminase. This is further supported by the fact that in band

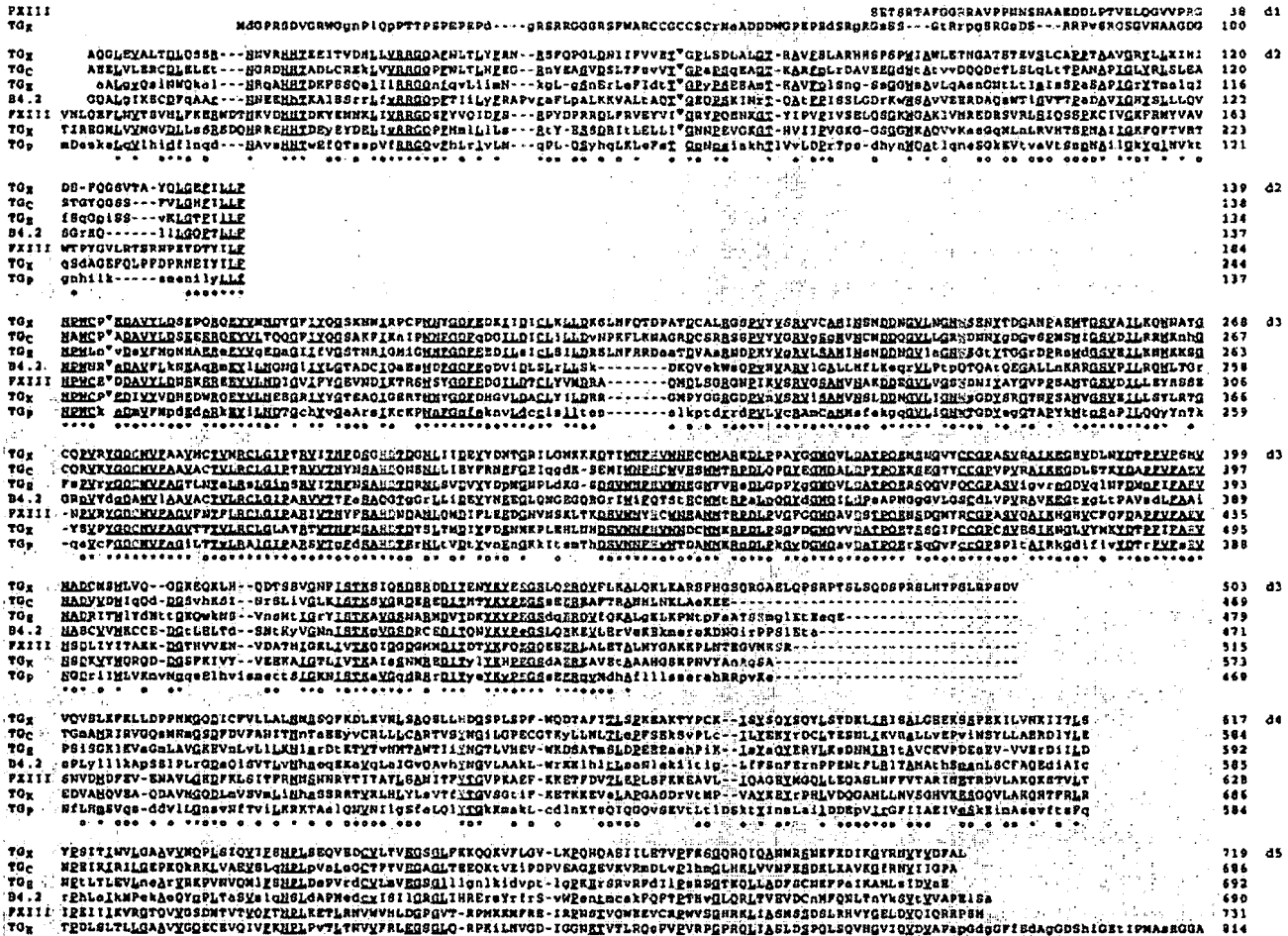

FIG. 7. **Comparison of the amino acid sequence of human $TG_X$ with the sequences of the other members of the transglutaminase family: $TG_C$, $TG_E$, band 4.2 protein, factor XIII a-subunit, $TG_K$ and $TG_P$.** The sequences are arranged to reflect the transglutaminase domain structure based on the crystal structure of factor XIII a-subunit (50, 52): N-terminal propeptide domain (*d1*), β-sandwich domain (*d2*), catalytic core domain (*d3*), and β-barrel domains 1 (*d4*) and 2 (*d5*) (from *top* to *bottom*). Human sequences are shown with positions of known amino acid variation between species denoted as small letters (for $TG_C$ (14), $TG_E$ (11), and band 4.2 protein (30, 54), human and mouse sequences were considered; for $TG_K$ (31, 40) and $TG_P$ (28, 41, 42), human and rat sequences were considered). *Dashes* indicate gaps inserted for optimal sequence alignment, *underlined* residues represent amino acids conserved in at least four gene products. *Asterisks* and *open circles* at the *bottom* of the aligned sequences indicate positions that are occupied by identical or chemically similar (57) amino acids in all transglutaminases. The active site Cys residue is shown in *red*, and the His and Asp residues of the catalytic triad are in *pink*. Additional residues involved in substrate interaction are shown in *light blue*. Residues involved in $Ca^{2+}$-binding are shown in *dark blue*, and protease cleavage sites in factor XIII a-subunit ($Arg^{37}$ and $Lys^{513}$), $TG_E$ ($Ser^{469}$), and $TG_K$ ($Arg^{90}$ and $Arg^{570}$) are marked in *green*. The alternatively spliced sequence in $TG_X$ and the known splice junctions for exons II/III and III/IV in the other transglutaminase gene products are marked with *arrowheads*.

TABLE IV

*Similarity of $TG_X$ to the other transglutaminase gene products in the individual domains*

The domain structure is based on the X-ray crystallographic structure of the factor XIII a-subunit dimer (50, 52) and inferred on the other gene products based on the sequence alignment shown in Fig. 7. The numbers reflect percent sequence identity.

| Gene product | Protein Domains | | | |
|---|---|---|---|---|
| | β-Sandwich | Catalytic core | β-Barrel 1 | β-Barrel 2 |
| $TG_C$ | 35.3 | 55.6 | 20.2 | 31.4 |
| $TG_E$ | 29.5 | 56.5 | 31.6 | 38.2 |
| B4.2 | 33.1 | 41.1 | 16.7 | 23.5 |
| FXIIIa | 23.7 | 46.8 | 18.4 | 25.5 |
| $TG_K$ | 25.9 | 49.6 | 18.4 | 29.4 |
| $TG_P$ | 23.7 | 47.1 | 11.4 | 20.6 |

4.2 protein, which is the only member of this protein family without catalytic activity, the residues directly involved in the cataytic process are not conserved (Fig. 7). The induction of $TG_X$ in differentiating keratinocytes further suggests that it might play a role in the formation of the cornified envelope. However, expression of $TG_X$ is not restricted to keratinocytes (Fig. 5), and further work is required to substantiate and extend the present findings.

*Conclusions*—Using the degenerate oligonucleotides, we have been able to amplify 5 out of 6 previously characterized transglutaminases and the novel transglutaminase $TG_X$. We have not been able to detect $TG_E$ which is likely due to its very restricted expression in the late stages of keratinocyte differentiation, particularly in hair follicles (11). Consistent with our observation, Kim *et al.* (11) reported that expression of $TG_E$ was not detectable in human keratinocyte cultures. Besides the expected type of transglutaminase, which turned out to be the predominant type of transglutaminase in the analyzed cell types, we detected other, apparently less abundantly expressed transglutaminases (Fig. 1, *A* and *C*). The abundance of the PCR product for a particular type of transglutaminase correlated with its message level detected in Northern blotting (compare

Fig. 1*D* and Fig. 4), and the sum of the PCR products for all transglutaminases (Fig. 6) correlated with the measured transglutaminase activity (see Ref. 25), at least on a semi-quantitative basis. These results suggest that the described degenerate oligonucleotides provide an excellent tool for identifying the types of transglutaminase expressed in a particular cell type and for cloning of new members of this growing gene family. The homology between vertebrate and invertebrate transglutaminases is similar to the different human transglutaminases compared with each other (3), indicating that these primers may work in a wide range of different species.

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